A TAXONOMIC STUDY OF THE GENUS CAMPYLOBACTER

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(ABSTRACT)

One hundred and eighteen (118) Campylobacter strains were studied by DNA homology experiments and characterized phenotypically. These strains formed eleven (11) distinct DNA homology groups (species) corresponding to C. fetus, C. "hyointestinalis", C. jejuni, C. coli, C. laridis, C. nitrofigilis, C. sputorum, C. mucosalis, C. concisus, and two unnamed groups currently referred to as the aerotolerant campylobacters and the "catalase-negative or weak" (CNW) strains. For practical reasons, we propose retaining the subspecies fetus and venerealis designations for C. fetus. In addition, we propose that the subspecies sputorum and bubulus designations for C. sputorum be dropped and replaced with biovars sputorum, bubulus and fecalis, the latter biovar including the catalase-positive strains formerly known as C. "fecalis". Biotyping schemes are also presented for C. jejuni and C. coli.

Growth at 25 and 42°C, sensitivity to nalidixic acid and cephalothin, growth in semisolid medium containing 1% glycine, 1% oxgall or 3.5% NaCl, growth in a semisolid minimal medium (MM), anaerobic growth in 0.1% trimethylamine-N-oxide (TMAO), H_2S production in Sulfide-Indole-Motility (SIM) medium, or on triple sugar iron (TSI) agar slants, hippurate hydrolysis, aerobic growth on agar plates, a requirement for H_2 or formate for

microaerophilic growth or H_2 or formate and fumarate for anaerobic growth, alkaline phosphatase activity, and deoxyribonuclease (DNase) activity proved to be the most useful phenotypic characteristics for identifying these strains at the species, subspecies and biovar levels.

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Introduction

Members of the genus Campylobacter have been recognized as important pathogens in veterinary medicine for over 60 years, primarily as causes of infertility and abortion in cattle and sheep. In the last decade, however, their importance in human medicine has also come to light. Today, Campylobacter jejuni and C. coli are considered major causes of gastroenteritis in man and they are being isolated as frequently as both Salmonella and Shigella species combined. Even though the importance of these organisms as human pathogens is now recognized, the epidemiology of campylobacter-associated enteritis is poorly understood. Many laboratories worldwide are involved in research aimed at a better understanding of the means by which this disease is transmitted.

There are two properties of campylobacters that have made taxonomic studies of these organisms difficult. First, they do not attack sugars, either fermentatively or oxidatively; and they are negative for most of the biochemical tests routinely used for identifying pathogenic bacteria. Therefore, speciation has been based on a relatively few biochemical and physiological tests and the reliability of these tests has often been questionable.

In recent years, a number of newly described species have been added to the genus Campylobacter, several of which may be pathogens and one species which is a free-living saprophyte. The great number of organisms that are currently being called campylobacters raises the question as to whether all of these organisms are highly related enough to be placed into the same genus.

The specific objectives of this research were:

1. To determine how many species exist within the genus Campylobacter based on the level of intraspecies and interspecies genetic relatedness determined by means of DNA homology experiments.

2. To determine what biochemical and physiological characteristics are the most reliable for accurately identifying these organisms at the species and subspecies levels by comparing assignment to species by DNA homology with assignment to species by conventional biochemical or physiological characteristics.

3. To expand the list of phenotypic characteristics that can be used to differentiate these organisms.

Literature Review

The genus Campylobacter consists of small, Gram-negative, curved, rod-shaped bacteria which have a characteristic "corkscrew" type of motility. Campylobacters are microaerophilic and most strains grow best under an atmosphere of 3-6% O_2 and 5-10% CO_2. Some species require either H_2 or formate as an electron donor for growth, and other species, although not requiring H_2, may be stimulated by its presence (Smibert 1984). Some species are able to grow anaerobically in the presence of an alternate electron acceptor (i. e. fumarate or nitrate), whereas other species cannot (Goodman and Hoffman 1983; Razi et al. 1981; Smibert 1984; Véron et al. 1981). Campylobacters do not ferment or oxidize sugars. Amino acids and tricarboxylic acid (TCA) cycle intermediates are their primary carbon and energy sources (Smibert 1984).

Vibrio fetus subspecies venerealis and V. fetus subspecies intestinalis. The association of campylobacters with animal disease was first recognized in the early 1900's. McFadyean and Stockman (1913) isolated a vibrio-shaped organism while studying an epidemic of abortion in sheep. In 1918, Smith and Taylor isolated an organism that they termed a spirillum from bovine abortions and concluded that this organism was the same as that previously described by McFadyean and Stockman (Smith and Taylor 1918). They subsequently named this organism Vibrio fetus (Smith and Taylor 1919). Later studies showed that there were actually two different types of animal fertility problems caused by Vibrio fetus and that there appeared to be two slightly, but distinctly different organisms involved (Bryner et al. 1964; Clark 1971; Florent 1959; Plastridge et al. 1947). One type of infection only affected cattle and was spread by sexual contact. This condition was termed

infectious infertility. Infected cows failed to become pregnant and soon returned to heat after being serviced by the bull. Very few actual abortions took place, because few of the pregnancies progessed that far. Cows became infected after sexual contact with an infected bull and the causative organism was not easily cleared from their reproductive tracts. The second type of infection, termed sporadic abortion, was spread by the ingestion of contaminated materials and affected both cattle and sheep. In this case, pregnant cows or ewes ingested the organisms, the organisms entered the bloodstream and then invaded the reproductive tract from the blood. Upon entering the reproductive tract, inflammation occurred, cutting off the oxygen supply to the fetus, resulting in abortion. After the abortion, these organisms were rapidly cleared from the reproductive tract of the infected animal (Dekeyser 1984). The organism responsible for infectious infertility was given the name Vibrio fetus subsp. venerealis, whereas the agent responsible for the sporadic form of vibrionic abortion was termed Vibrio fetus subsp. intestinalis (Florent 1959). V. fetus subsp. venerealis could be distinguished from V. fetus subsp. intestinalis by the inability of the former to grow in the presence of 1% glycine (Florent 1959; Lecce 1958). Another important distinguishing characteristic was discovered by Bryner and colleagues in the 1960's (Bryner et al. 1964). They found that V. fetus subsp. venerealis was unable to survive in the intestinal tracts of several different types of animals, including cattle and sheep. However, V. fetus subsp. intestinalis was able to survive for a number of days. This explained why V. fetus subsp. venerealis was only transmitted via the venereal route (Bryner et al. 1964).

In the late 1940's, Vinzent et al. (1947) reported the isolation of a Vibrio fetus strain from the blood of a human patient suffering from septicemia. Since that time, it has been shown that the same organisms responsible for sporadic abortion in cattle and sheep, now called Campylobacter fetus subsp. fetus (Véron and Chatelain 1973), can cause serious bloodstream infections in immunocompromised humans (Guerrant et al. 1978; Mandal et al. 1984; Rettig 1979). The organism responsible for the infectious abortion in cattle, now called C. fetus subsp. venerealis (Véron and Chatelain 1973) is not considered to be a human pathogen (Rettig 1979).

Vibrio jejuni. Smith and Orcutt (1927) isolated a vibrio-shaped organism from winter scours in cattle, and Jones and colleagues later named this organism Vibrio jejuni (Jones and Little 1931a, 1931b; Jones et al. 1931). Organisms resembling V. jejuni were later found to cause abortion in sheep (Bryans et al. 1960; Smibert 1978). E. O. King (1957) isolated similar organisms from the blood of patients suffering from diarrhea. She called these organisms "related vibrios" because they had many phenotypic characteristics in common with V. fetus but were able to grow well at 42°C, but not at 25°C, which was not characteristic of V. fetus. V. fetus grew well at 25°C, but would not grow at 42°C. The "related vibrios" were also serologically different. King suspected that the "related vibrios" played an important role in human gastroenteritis, but she was never able to isolate these organisms from fecal samples. It was not until Dekeyser and colleagues in Belgium used filtration and a selective medium to isolate the first "related vibrios" from the feces of a patient suffering from gastroenteritis (Dekeyser et al. 1972) that this role for these organisms was proven.

Vibrio coli. Doyle (1944) isolated an organism from swine dysentery which he later named V. coli (Doyle 1948). Similar organisms were later isolated from swine intestines and feces (Davis 1961; Lussier 1962; Soderlind 1965), but these organisms were positive for nitrate reduction, whereas the original V. coli culture was unable to reduce nitrate (Doyle 1944). These organisms were very similar to the "related vibrios" of King in their phenotypic characteristics and some workers in the field considered them to be the same organism (Smibert 1978).

Vibrio sputorum subsp. sputorum, V. sputorum subsp. bubulus and V. fecalis. Florent (1953) isolated vibrios from the bovine reproductive tract that resembled V. fetus, but were catalase-negative, seemed to be more anaerobic in nature than V. fetus and produced much more H_2S than did V. fetus. He called this organism V. bubulus. V. bubulus was considered to be normal genital flora in cattle. Bryner and Frank (1955) later suggested that the catalase test was a good means of differentiating the animal pathogens from the commensals, the pathogens being catalase-positive and the commensals catalase-negative. An organism similar to V. bubulus had been isolated earlier from a case of human bronchitis by Tunicliff (1914) and later named V. sputorum by Prévot (1940). Loesche and colleagues (1965) performed a study of catalase-negative vibrios isolated from the human mouth and concluded that these strains were not sufficiently different from strains of V. bubulus in their phenotypic characteristics to warrant being placed into a different species. They proposed the name V. sputorum subspecies sputorum for the human oral strains and V. sputorum subspecies bubulus for the strains from the bovine reproductive tract. Firehammer (1965) isolated a catalase-positive

vibrio from the feces of normal sheep, which he called V. fecalis. However, in all of its phenotypic characteristics except catalase activity this organism was identical to V. sputorum subspecies bubulus.

Creation of the genus Campylobacter. In 1963, Sebald and Véron reported that the mol% G + C of the DNA from V. bubulus and V. fetus was 30 and 34, respectively. This is considerably lower than the mol% G + C values they found for other Vibrio species (ca. 47). They also noted that although the classical Vibrio species were facultative, V. bubulus and V. fetus were only capable of a respiratory type of metabolism. For these reasons, they proposed that V. bubulus and V. fetus be placed into a new genus, Campylobacter (Cam.py' .lo.bac.ter. Gr. adj. campylo curved; Gr. n. bacter rod; M.L. masc. n. Campylobacter a curved rod). In 1973, Véron and Chatelain published a critical study of these organisms and proposed the following species (see Table 1): Campylobacter fetus subsp. fetus for the organism that caused sporadic abortion in cattle and sheep, C. fetus subsp. venerealis for the causative agent of venereally transmitted abortion unique to cattle, C. coli for the strains isolated from porcine intestines and some of King's "related vibrios", C. jejuni for the strains formerly classified as V. jejuni, C. sputurom subsp. sputorum for the human oral catalase-negative strains and C. sputorum subsp. bubulus for the catalase-negative strains from bovine genitals. They did not study any V. fecalis strains, but they stated that strains previously described as V. fecalis were possibly C. coli strains in reality.

Significance of campylobacters in human gastroenteritis. The recognition of C. jejuni as a major cause of diarrheal disease in man came in the 1970's.

Table 1. Comparison of old names for the campylobacters with the names assigned by Véron and Chatelain (1973).

Old nomenclature	Nomenclature of Véron and Chatelain (1973)
Vibrio fetus subsp. *intestinalis*	*Campylobacter fetus* subsp. *fetus*
Vibrio fetus subsp. *venerealis*	*Campylobacter fetus* subsp. *venerealis*
Vibrio jejuni	*Campylobacter jejuni*
Vibrio coli	*Campylobacter coli*
Vibrio sputorum	*Campylobacter sputorum* subsp. *sputorum*
Vibrio bubulus	*Campylobacter sputorum* subsp. *bubulus*

As mentioned previously, in 1972, Dekeyser and associates isolated "related vibrios" from the stools of two patents suffering from enteritis (Dekeyser et al. 1972). Between 1944 and 1967 a number of other cases of human disease due to this organism had been reported (Darrell et al. 1967; Levy 1946; Middlekamp and Wolf 1961; Wheeler and Borchers 1961; White 1967), but all of the previous isolations had been from blood. One reason that these organisms had not been recognized earlier as causes of enteritis may have been that cultures from fecal specimens were not routinely incubated under microaerobic conditions. Overgrowth of the campylobacters by normal fecal flora may have been another problem. The successful isolation of "related vibrios" by Dekeyser et al. was partly due to the use of a selective filtration technique to aid in the isolation of these organisms (Dekeyser et al. 1972). This technique was based on the fact that the campylobacters were considerably smaller than most of the bacteria encountered as normal flora in feces. Thus, filters (0.65 μm) were used that had pore sizes small enough to retain the normal flora but allow the campylobacters to pass through. Dekeyser and colleagues also plated their filtrate on a medium containing antibiotics, which suppressed part of the normal fecal flora. Several investigators subsequently developed selective media for the campylobacters (Blaser et al. 1979; Butzler and Skirrow 1979; Skirrow 1977) and clinical laboratories began to incubate cultures from fecal specimens suspected of containing campylobacters at 42°C. C. jejuni and C. coli grow faster at this temperature than they do at 37°C, whereas most of the enteric bacteria either do not grow at all or grow only poorly at 42°C (Goossens et al. 1984). The combination of filtration of the feces, plating on selective media, and

incubating the plate under microaerobic conditions at 42°C, resulted in an increased isolation rate from feces. By the end of the 1970's, medical microbiologists began to realize just how important these organisms are as causes of human gastroenteritis (Blaser et al. 1979; Blaser 1980, Blaser and Reller 1981, Skirrow 1977). C. jejuni and C. coli are now being isolated as often as both Salmonella and Shigella species (Blaser and Reller 1980, Smibert 1984).

Pathogenic mechanisms in campylobacter-associated gastroenteritis. The exact means by which campylobacters cause gastroenteritis is unknown. The campylobacters are invasive (Butzler and Skirrow 1979; Ruiz-Palacios et al. 1981) and it was originally believed that this was their primary means of pathogenicity. However, in 1983, Ruiz-Palacios reported finding a cholera-like enterotoxin from C. jejuni (Ruiz-Palacios et al. 1983). This enterotoxin caused fluid accumulation in a ligated ileal loop from a rat in the same way as cholera toxin, but not in a rabbit ileal loop. Antisera against cholera toxin was able to block the action of the C. jejuni toxin in the rat ileal loop system. Even though this cholera-like toxin is probably an important virulence factor, it does not appear to be necessary for virulence (Ruiz-Palacios et. al 1983). This probably explains why there appear to be two distinctly different types of enteritis caused by C. jejuni. One type presents itself in the form of bloody stools, which is similar to the diarrhea caused by Shigella species and is indicative of an invasive organism. The second type is more like the diarrhea produced by V. cholerae, which consists of watery stools and suggests the presence of an enterotoxin. There may also be geographical differences in the relative distribution and importance of these different

strains. The majority of the cases of campylobacter-associated gastroenteritis in industrialized countries appear to be caused by the invasive strains, the symptoms most commonly seen include the bloody stools indicative of an invasive type of diarrhea. On the other hand, many of the cases of diarrhea caused by Campylobacter species in developing countries present symptoms that indicate the presence of an enterotoxin-producing organism, i. e. watery, profuse stools (Mandal et al 1984). At present, there is no good animal model for studying the pathogenesis of C. jejuni or C. coli (Newell 1984). Several species of animals have been looked at in this regard, but none have proven completely successful. Such a model would be very useful for determining the exact mechanisms that are responsible for the pathogenicity of the organisms and the relative importance of each.

Epidemiology of campylobacter-associated gastroenteritis. Although the importance of the campylobacters as human intestinal pathogens is now recognized, the epidemiology of campylobacter-associated gastroenteritis is not well understood. C. jejuni can be isolated from cattle, sheep, dogs, poultry, wild birds and other wild animals and zoo animals (Luechtefeld and Wang 1982; Smibert 1984). C. coli is isolated mainly from pigs, but can also be found in poultry (Luechtefeld and Wang 1982) and occasionally in dogs (Ursing et al. 1983). Thus it appears that these organisms occupy different ecological niches. For this reason, it is essential that the strains be properly identified to the species level. This has presented a problem over the years, and these organisms were often identified under the names C. fetus subsp. jejuni (Smibert 1978) or C. jejuni/coli. One reason for this was that until Skirrow and Benjamin (1980b) found that C. jejuni and C. coli differed in

their reactions in the hippurate hydrolysis test, there was no reliable biochemical test available to distinguish between them. The lumping together of these organisms makes the epidemiological picture in the 1970's cloudy at best.

Campylobacter-associated gastroenteritis is believed to be transmitted by the ingestion of contaminated materials and direct human to human transmission is not thought to be important (Skirrow 1982). Poultry has been implicated as a major source of infections (Blaser et al. 1984; Skirrow 1982), and this has been the subject of a great deal of research. Poultry appear to carry both C. jejuni and C. coli as commensals (Luechtefeld and Wang 1982; Skirrow 1982; Smibert 1984). These bacteria have been isolated from the intestinal material of the majority of birds in a slaughter house (Luechtefeld and Wang 1982; Wempe et al. 1983). Also, C. jejuni and C. coli have been isolated from a high percentage of the raw chickens in grocery stores (Grant 1980; Park et al. 1981; Simmons and Gibbs 1979).

In addition to properly identifying C. jejuni and C. coli at the species level, other epidemiological markers are needed to determine how campylobacter enteric infections are transmitted. Several biotyping schemes have been developed (Bolton et al. 1984; Hébert et al. 1982; Lior 1984; Skirrow and Benjamin 1980b). Skirrow and Benjamin (1980b) developed one that divides C. jejuni into two biotypes, based on the ability of strains to produce H_2S in an iron-based "sensitive" medium. This scheme is the one that is most commonly used and it appears that biotype 1 strains are more prevalent than biotype 2 strains (Skirrow and Benjamin 1982). Several serotyping systems have also been developed (Abbott et al. 1980; Lauwers et

al. 1981; Lior et al. 1982; Penner and Hennessy 1980). The two most widely used are those of Penner and Hennessy (1980) and Lior et al. (1982). These schemes have proven very useful in studying the epidemiology of these organisms (Blaser et al. 1982; Blaser et al. 1983; Chan et al. 1984; Karmali et al. 1983; Lastovica and Penner 1983; McMyne et al. 1982; Munroe et al. 1983; Penner et al. 1983a; Penner et al. 1983b; Vogt et al. 1984), but the reagents for these schemes are not currently commercially available. Plasmid fingerprinting and bacterial restriction endonuclease DNA analysis (BRENDA) have also been used for studying the epidemiology of C. jejuni and C. coli (Kakoyiannis et al. 1984; Tenover et al. 1984), but are not of practical use for most clinical microbiology laboratories.

C. sputorum subsp. mucosalis. In 1974, Lawson and Rowland isolated Gram-negative, curved bacteria from the lesions of porcine intestinal adenomatosis (PIA) and called this organism C. sputorum subsp. mucosalis, because it was catalase-negative and produced rather large amounts of H_2S (Lawson and Rowland 1974; Lawson et al. 1975). However, this organism differed from the other two subspecies of C. sputorum (C. sputorum subsp. sputorum and C. sputorum subsp. bubulus) by having a requirement for either H_2 or formate as an electron donor for growth (Lawson et al. 1981). Similar organisms have also been isolated from the mouths of pigs (Lawson and Rowland 1984). C. sputorum subsp. mucosalis was originally thought to be the causative agent of PIA (Lawson and Rowland 1974), but attempts to reproduce PIA in infectivity studies have proven unsuccessful (Lawson and Rowland 1984; McCartney et al. 1984; Roberts et al. 1980). Recent reports

suggest that another campylobacter may also be involved (Gebhart et al. 1983; McCartney et al. 1984).

Aerotolerant campylobacters. Neill and Ellis (1977) isolated organisms resembling Campylobacter species from porcine and bovine abortions using Leptospira enrichment medium (EMJH medium); however, these organisms differed from the other campylobacters in that they were able to grow aerobically upon subculture (Ellis et al. 1977; Ellis et al. 1978; Neill et al. 1978; Neill et al. 1979; Neill et al. 1980). Thus these organisms have been termed "aerotolerant campylobacters". Similar organisms have since been isolated from ovine and equine abortions (Hanna et al. 1983), as well as from cases of mastitis in cows (Logan et al. 1982). Electrophoretic patterns of acid-phenol extracts from these organisms have indicated that these organisms are not related at the species level to reference strains of C. fetus subsp. fetus, C. fetus subsp. venerealis, C. jejuni, C. coli, C. sputorum subspp. sputorum, C. sputorum subsp. bubulus or C. sputorum subsp. mucosalis (Hanna et al. 1983).

C. laridis. Skirrow and Benjamin (1980a) described a group of campylobacters that were very similar to C. jejuni and C. coli in their phenotypic characteristics except that these new strains were resistant to nalidixic acid. The first isolation of these new strains was from the feces of a symptomless child, but the majority of these organisms have been isolated from wild seagulls of the genus Larus (Benjamin et al. 1983). Skirrow and Benjamin (1980a) originally called these organisms the NARTC (nalidixic acid-resistant thermophilic Campylobacter) strains and later proposed the name C. laridis (Benjamin et al. 1983). These organisms have also been

isolated from dogs, monkeys, cattle, and a foal, and from water samples (Benjamin et al. 1983). Only recently have they been implicated in human disease (Nachamkin et al. 1984).

C. nitrofigilis. A microaerophilic, nitrogen-fixing, curved bacterium was isolated from the surface-sterilized roots of Spartina grass by McClung and Patriquin (1981). This organism had many of the phenotypic characteristics associated with Campylobacter species and was later given the name C. nitrofigilis (McClung et al. 1983). This organism has an NaCl requirement and was isolated from a symbiotic relationship with a plant, neither of which is characteristic of other Campylobacter species.

C. concisus. Tanner et al. (1981) reported isolating a catalase-negative campylobacter from the mouths of humans suffering from periodontal disease. This organism showed many of the phenotypic characteristics that are associated with C. sputorum strains, but DNA homology experiments showed that this organism was not related to either C. sputorum subsp. sputorum or subsp. bubulus at the species level. This organism required either H_2 or formate as an electron donor for growth, as does C. sputorum subsp. mucosalis. However, C. concisus and C. sputorum subsp. mucosalis strains were not compared in the previously mentioned DNA homology studies (Tanner et al. 1981). The role of this organism in periodontal disease is not known.

"C. hyointestinalis". Campylobacters have been isolated from pigs suffering from proliferative ileitis (Gebhart et al. 1983). These organisms most closely resembled C. fetus subsp. fetus in their phenotypic characteristics, but were unlike C. fetus in that they produced H_2S on triple sugar iron (TSI) agar slants. They were given the name "C. hyointestinalis". Similar organisms

have since been isolated from both healthy and diarrheic cattle (Myers et al. 1984; Ursing et al. 1983).

Other campylobacters. Catalase-negative campylobacters with phenotypic characteristics most closely resembling C. coli (catalase-positive) strains have been isolated from dogs suffering from diarrhea and healthy dogs by a group in Sweden (Sandstedt et al. 1983). DNA homology studies showed that these organisms were not highly related to C. fetus, C. coli, C. jejuni or C. sputorum reference strains. More recently, other investigators isolated similar organisms from both healthy and diarrheic dogs and cats in the U. S. (Gebhart et al. 1984; Davies et al. 1984). What role, if any, these organisms play in enteritis in dogs or cats is at present unknown.

"Campylobacter-like organisms", also termed "CLO's", were isolated from rectal cultures from homosexual men by Fennell et al. (1984). These organisms showed many of the characteristics associated with the genus Campylobacter, but could not be placed into any of the currently recognized species. Initial DNA homology studies using radiolabeled DNA probes from these organisms have shown that they are not related at the species level to C. fetus, C. coli, C. jejuni or "C. fecalis". It was also found that the CLO's could be divided into three distinct DNA homology groups (Fennell et al. 1984). More extensive DNA homology studies have shown that these organisms can be divided into 4 DNA homology groups (P. Totten, personal communication). A potential role of these organisms in proctocolitis in homosexual men has been suggested (Fennell et al. 1984) but has not been proven. CLO's have also been isolated from bacteremia in two immunocompromised homosexual men with tuberculosis (Pasternak et al. 1984).

Recently, investigators from Australia reported finding campylobacter-like bacteria in almost all of the cases of gastritis they studied. These organisms appeared to be present in quite large numbers, as they were visible upon examination of biopsy slides under light microscopy (Warren and Marshall 1983). They have been termed the "pyloric campylobacters". They are microaerophilic, Gram-negative, short spirals, 0.5 μm by 2.5 μm, with one or two helical turns and with up to five sheathed flagella on one end. They grow only at 37°C, are catalase- and oxidase-positive and sensitive to a number of antibiotics, including metronidazole and nalidixic acid (Jones et al. 1984; Rollason et al. 1984). They have also been shown to have some serological cross-reactivity with C. jejuni (Jones et al. 1984). What role, if any, they play in gastritis is at present unknown.

DNA homology experiments in bacterial taxonomy. In the last 20 years, the development of techniques for measuring DNA homology have provided investigators with a powerful tool for determining the genetic relatedness of bacteria. However, since these techniques compare the entire genome, only bacteria with a high degree of base sequence similarity (ca. 90% or more) will show any significant level of DNA homology. Consequently, these techniques are mainly useful for determining genetic relationships at the species level (Johnson 1984).

There are several different methods that are used for determining DNA homology. Single stranded (denatured) DNA from an organism can be bound to a nitrocellulose membrane and the membrane subsequently treated so that no more DNA can bind to the membrane. DNA homology can then be measured by

allowing radioactively-labelled, single stranded DNA to hybridize with the DNA on the membrane under suitable conditions of temperature, pH and salt concentration for an appropriate length of time (usually 24 h). The amount of heterologous DNA binding to the membrane is then compared to the amount of homologous DNA that has been bound to determine the level of homology. This is known as the direct binding method (Johnson 1981). In a variation of this procedure, a large excess of heterologous or homologous, unlabelled DNA ("competitor" DNA) is incubated with a small amount of labelled DNA homologous to the DNA bound to the membrane. The degree to which the unlabeled DNA is able to compete with labeled DNA is used as a measure of DNA homology. This is known as the membrane-filter competition method (Johnson 1981).

Free solution hybridization is also used to measure DNA homology. This method is based on the fact that under the proper conditions of temperature, pH and salt concentration, single-stranded DNA will reassociate into duplexes at a rate directly proportional to the concentration of DNA present in the reaction mixture. A small amount of labeled DNA is incubated with a vast excess of unlabeled DNA. After an incubation period of 24 h, if the two DNA's share a high degree of base pair similarity, most of the labeled DNA will be in duplex form. The amount of heteroduplex formation is compared to the amount of homoduplex formation to determine the level of DNA homology. There are two popular ways to determine the amount of duplex formation after the incubation period. In the S-1 nuclease procedure, the incubation mixture is treated with deoxyribonuclease S-1, isolated from Asperigillus oryzae, which under proper conditions specifically hydrolyzes single-stranded

DNA. After this treatment, the remaining double-stranded DNA is precipitated with trichloroacetic or hydrochloric acid, collected on nitrocellulose membranes and the radioactivity counted. In the hydroxyapatite (HA) procedure, the incubation mixture is placed on an HA column or mixed with HA in a batch procedure. Double-stranded DNA binds to HA most strongly at a phosphate concentration of 0.14 M. In the HA procedure, after the double-stranded DNA has bound to the HA and the single-stranded DNA has been removed by washing the column or centrifuging and washing the batch, the double-stranded DNA can be eluted from the HA by increasing the phosphate concentration to 0.28 M. This DNA is then precipitated by acid and counted as described above (Johnson 1981). An optical method has also been used for measuring DNA homology. In this procedure, the renaturation of two single-stranded DNA preparations is monitored with a recording spectrophotometer. A mixture containing single-stranded DNA from two organisms which share similar base pair sequences will reassociate at a rate very similar to the rate of the DNA from one of the strains alone. If their is less sequence identity, the rate will be slower (Schleifer and Stackenbrandt 1983).

Comparison of homology results obtained using different methods. It is important to know which method has been used when analyzing DNA homology data. The results obtained from different methods often cannot be directly compared. For example, the S-1 nuclease method yields consistently lower DNA homology values than do the other methods, especially at DNA homology values lower than 50%, even if the same strains are employed (Coykendall and Munzenmaier 1978, Grimont et al. 1980; Johnson 1984). This is due to the

fact that only true hybrids remain intact after S-1 treatment, whereas in the other methods both hybrids and "tails" resulting from unmatched single-stranded labeled DNA are detected. It is also important to know the temperature of incubation used. Some investigators use 25°C below the thermal melting point (T_m) of the DNA, which is termed the "optimal" temperature for hybridization (Johnson and Ordal 1968; Marmur and Doty 1961). Johnson and Ordal (1968) showed that at the optimal temperature the heteroduplexes formed from single-stranded DNA from highly related organisms exhibited a thermal stability very similar to that of native DNA, but at temperatures below this considerable renaturation would occur between single-stranded DNAs from organisms which were not highly related, but the thermal stability of the resulting heteroduplexes was much lower than that of native DNA. Other investigators use use both "optimal" conditions and an incubation temperature of 15°C below the T_m of the DNA, which is sometimes termed "stringent" conditions (Schleifer and Stackenbrandt 1983). "Stringent" conditions are useful for determining the level of base-pair mismatching between DNA hybrids (Johnson and Ordal 1968). This is especially useful in cases were the DNA homology values obtained under "optimal" conditions are considered borderline for species identity, i. e., 40 to 50% DNA homology using the S-1 procedure. Another method of determining the amount of base-pair mismatching between DNA hybrids is to perform a thermal melting profile of the hybrid DNA. If the T_m of the hybrid is very much lower than that of the native DNA of the reference strain, this is an indication that there is considerable base-pair mismatching. Estimates range from 1% to 2.2%

base-pair mismatching for each °C that the T_m of the hybrid is lowered (Johnson 1981).

Labeling DNA for homology experiments. The type of radioisotope used for labelling DNA for homology experiments varies among investigators. Some use tritiated nucleotides and label the DNA in vitro using the "nick-translation" procedure. In this procedure, the DNA is nicked by a DNase and a DNA polymerase is used to reconstruct part of the DNA using the added radioactive nucleotides (Johnson 1981). Radioactive iodine (^{125}I) can be used in an in vitro procedure. In this procedure, the radioactive iodine bonds covalently to the cytosine residues of the DNA in the presence of a thallium catalyst (Commerford 1971; Selin et al. 1983; Tereba and McCarthy 1973). A third method involves growing the organisms from which the reference DNA is to be isolated in the presence of ^{32}P (Johnson et al. 1981).

DNA homology studies of campylobacters. Several groups have performed DNA homology studies on catalase-positive campylobacter strains (Belland and Trust 1982; Benjamin et al. 1983; Harvey and Greenwood 1983; Hébert et al. 1983; Leaper and Owen 1982; Owen 1983; Owen and Leaper 1981; Ursing et al. 1983). Owen and Leaper (1981) performed a limited study using labelled DNA from a reference strain of C. coli and determined that C. coli was not related to C. jejuni, C. fetus or a NARTC strain at the species level. Later studies by these and other groups showed that C. fetus, C. jejuni, C. coli, C. laridis and "C. fecalis" were distinct species. In addition, two of these groups (Belland and Trust 1982; Ursing et al. 1983) used both "optimal" and "stringent" conditions to show that although C. jejuni and C. coli strains were more highly related to each other than they were to the other

catalase-positive campylobacters, there was considerable base-pair mismatching in the heteroduplexes formed by the reassociation of DNA from these two organisms. This finding strenghtened the argument that C. jejuni and C. coli are separate species.

Although many of the species relationships within the genus Campylobacter have been defined by means of DNA homology experiments, there are still others that need clarifying, such as the relationship between "C. hyointestinalis" and C. fetus, the relationship of C. sputorum subsp. mucosalis to C. sputorum subsp. sputorum, C. sputorum subsp. mucosalis to C. concisus, etc. Also, it would be useful to know which of the phenotypic tests used to differentiate these organisms provide the most reliable identification at the species and subspecies levels and if possible to expand the number of tests available for differentiating these organisms. This is particularly true in cases where only a single phenotypic test is presently available for differentiating between species, i. e., C. jejuni vs. C. coli, or C. fetus subsp. fetus vs. "C. hyointestinalis".

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Chapter 1

Differential characteristics of catalase-positive campylobacters correlated with DNA homology groups

Abstract

Eighty-four strains of catalase-positive campylobacters could be placed into seven distinct DNA homology groups (species), corresponding to Campylobacter fetus, "C. hyointestinalis", C. jejuni, C. coli, "C. laridis", "C. fecalis", and "aerotolerant campylobacters". The biochemical and physiological characteristics of the strains were examined for their correlation with the homology groups. The characterization tests that provided the most reliable differentiation at the species and subspecies level were: growth at 25 and 42°C; sensitivity to cephalothin and nalidixic acid; growth in semisolid media containing 1% glycine and 3.5% NaCl; growth on plates containing 1.5% NaCl; growth in a semisolid minimal medium; anaerobic growth in the presence of 0.1% trimethylamine-N-oxide; hydrogen sulfide production in SIM medium and triple-sugar iron agar; hippurate hydrolysis; nitrite reduction; and growth on plates under an air atmosphere.

Introduction

The catalase-positive campylobacters are important pathogens in both human and veterinary medicine. Véron and Chatelain (1973) separated these organisms into three species: C. fetus, C. jejuni and C. coli, with C. fetus being divided into two subspecies, C. fetus ssp. fetus and C. fetus ssp. venerealis. C. jejuni is an important cause of gastroenteritis in man (Butzler et al. 1973; Skirrow 1977) and causes abortion in sheep (Smibert 1978). C. fetus ssp. fetus causes blood infections in debilitated patients (Rettig 1979) and is a causative agent of orally transmitted sporadic abortion in cattle, while C. fetus ssp. venerealis causes venereally transmitted abortion in cattle (Bryner et al. 1964; 1971), but is not a human pathogen (Rettig 1979). C. coli is usually isolated from normal pig feces (Deas 1960; Lawson and Rowland 1974), but has been isolated from cases of gastroenteritis in man (Leaper and Owen 1981; this study). "C. fecalis" can be isolated from sheep feces, but is not known to be pathogenic (Firehammer 1965).

Three new catalase-positive campylobacters have been described. "C. laridis", formerly referred to as NARTC ("nalidixic acid-resistant thermophilic Campylobacter"), was originally isolated from wild seagulls and is similar to C. jejuni and C. coli in its phenotypic characteristics. It has not been implicated in human or animal infection (Benjamin et al. 1983). "C. hyointestinalis" has been isolated from pigs with proliferative ileitis (Gebhart et al. 1983). A currently unnamed aerotolerant campylobacter has been isolated from aborted porcine, bovine, ovine and equine fetuses (Hanna et al. 1983).

Classification of bacteria based upon DNA homology experiments provides a stable and objective basis for speciation and several studies of this nature have been reported for the catalase-positive campylobacters (Belland and Trust 1982; Benjamin et al. 1983; Harvey and Greenwood 1983; Hébert et al. 1983; Leaper and Owen 1982; Owen and Leaper 1981; Owen 1983; Ursing et al. 1983). The results of such studies can lead to the subsequent determination of those phenotypic characteristics that can reliably differentiate the various species. Unfortunately, relatively few characteristics have been found to be useful for campylobacters in this regard. This report describes several biochemical tests which are correlated with DNA homology results and can differentiate the catalase-positive campylobacters.

Materials and methods

Organisms

The majority of the strains were obtained from the culture collection of one of the authors (RMS). The origins of all strains used in this study are listed in Table 1.

Growth conditions

Stock cultures were maintained in Brucella broth containing 0.16% agar (semisolid Brucella medium) at 37°C, with weekly transfer. Cultures were also preserved by freezing in liquid nitrogen.

For DNA isolation, each strain was inoculated into semisolid Brucella medium and incubated at 37°C (except for the aerotolerant campylobacters, which were cultured at 30°C) for 24 h. The top 1-2 mL of growth from two tubes were used to inoculate medium in a Roux bottle. These bottles contained a diphasic medium consisting of 200 mL of Brucella broth supplemented with ferrous sulfate, sodium bisulfite, and sodium pyruvate (FBP medium; George et al. 1978) solidified with 2.5% agar and overlayed with 50 mL of FBP broth. For culturing "C. fecalis", unsupplemented Brucella medium was used because of excessive blackening of the FBP medium due to H_2S production. After aerobic incubation for 24 h the growth harvested from four Roux bottles was used as inoculum for 2 L of FBP broth in a 4-L Erlenmeyer flask. The broth cultures were incubated aerobically with shaking or magnetic stirring until a heavy turbidity occurred (usually 24 h). For diphasic and broth cultures, the use of a large inoculum (ca. 10%,

v/v) eliminated the requirement for microaerobic conditions. Before harvesting, all cultures were checked for purity by phase-contrast microscopy and, in questionable cases, also by streaking onto FBP agar plates which were incubated under aerobic and microaerobic conditions.

DNA isolation and DNA homology experiments

DNA was extracted and purified by the hydroxylapatite (HA) procedure described by Johnson (1981). The S-1 nuclease procedure described by Johnson (1981) was used for the DNA homology experiments. Homology experiments were performed under "optimal" conditions, i.e., 25°C below the thermal melting point (T_m) of the DNA (Marmur and Doty 1961). DNA from reference strains was labeled in vitro with ^{125}I according to the method of Selin et al. (1983).

DNA base composition

The thermal melting point (T_m) of the DNA of representative strains from each homology group was determined using the method described by Johnson (1981) and the mole percent guanine plus cytosine (mol% G + C) was determined using the equation of Mandel et al. (1970). DNA from Escherichia coli strain b, which has a mol % G+C of 51, was used as a reference.

Physiological characteristics

Tests for nitrate and nitrite reduction, fermentation or oxidation of carbohydrates, indole production, catalase production, H_2S production in Sulfide-Indole-Motility (SIM) medium (Difco) or, by the lead acetate strip

method, from cysteine, growth at 25 and 42°C, growth in 3.5% NaCl and 1% glycine, and sensitivity to nalidixic acid were performed as described by Holdeman et al. (1977). Oxidase tests were performed using the methods described by Smibert and Krieg (1981). Growth in 1% bile was determined in semisolid Brucella medium supplemented with 1% Bacto-oxgall (Difco). This test was performed in the same way as the tolerance tests (i.e. 1% glycine) listed above. Sensitivity to cephalothin (30 µg disks) and metronidazole (5 µg disks) was determined after 48 h on Brucella agar plates. Unless otherwise indicated, all plates were incubated at 37°C under an atmosphere of 6% O_2, 5% CO_2 and 89% N_2. Strains showing any zone of inhibition were considered sensitive. Growth on Brucella agar plates supplemented with 2,3,5-triphenyltetrazolium chloride (TTC) (0.4 and 1.0 g/L) or brilliant green (1:33,000 and 1:100,000) was determined after incubation for 5 days. H_2S production within 7 days was tested on triple sugar iron agar (TSI; Difco) containing water of syneresis at the junction of the slant with the butt. The TSI slants were incubated at 37°C under an atmosphere of 6% O_2, 5% CO_2 and 89% N_2. Growth in 1.5% NaCl was tested according to the method of Benjamin et al. (1983), with the exception that Brucella agar was used instead of yeast extract-nutrient agar (YNA). The ability to grow anaerobically in 0.1% trimethylamine-N-oxide (TMAO) was tested using a modification of the technique described by Benjamin et al. (1983). Instead of yeast-extract nutrient broth (YNB), a medium of the following composition was used (g/L): Bacto-peptone, 10; Bacto-tryptone, 10; yeast extract, 1; NaCl, 5; agar, 2. Twenty-mL portions of this medium were dispensed into 20 x 150 mm screw-capped tubes and were freshly autoclaved or steamed and

cooled to 45°C immediately before use. The medium was inoculated with 4-6 drops from a Pasteur pipet of a 48-h-old semisolid culture; it was incubated at 37°C with the cap screwed down tightly and examined for growth occurring throughout the tube within 7 days. Growth only on the surface of the medium was considered a negative reaction.

The method described by Harvey (1980) was used for detecting hippurate hydrolysis. A dark purple color after incubation with ninhydrin for 10 min at 37°C was considered a positive reaction; a light blue color or no color was considered negative. For determining alkaline phosphatase and aminopeptidase activities, the cells were grown in diphasic culture in Roux bottles for 24 to 48 h, harvested by centrifugation at 13,000 x g for 10 min, and washed once with sterile 0.85% saline. The cell pellet was suspended in 0.85% saline and the cells were adjusted to a turbidity equivalent to a #3 McFarland standard. Alkaline phosphatase activity was determined using a modification of the method described by Hébert et al. (1982). One-half mL of the cell suspension was pipetted into 16 x 125-mm tubes, 0.5 mL of alkaline phosphatase reagent (100 mg p-nitrophenyl phosphate disodium tetrahydrate; 25 ml distilled water; 25 ml of 0.1 M glycine in 0.001 M $MgCl_2$, pH 10.5) was added and the color reaction observed after incubation at 37°C for 6 h. Any degree of yellow color was considered positive. C. jejuni strain 13136 served as a positive control and sterile saline was used as a negative control. Aminopeptidase activities were determined by inoculating 2 or 3 drops of the bacterial suspension from a Pasteur pipet into 0.4 mL of a 0.1% solution of the amino acid-ß-napthylamide in 0.1 M Tris buffer, pH 8.0 (D'Amato et al. 1978). After incubation at 37°C for 6 h, 0.2 mL of cinnamaldehyde reagent

was added. This reagent was based on the API 20S Streptococcus system, Analytab, New York and consisted of p-dimethylaminocinnamaldehyde, 0.15 g; sodium dodecyl sulfate, 25 g; 2-methoxyethanol, 50 mL; glacial acetic acid, 25 mL; and distilled water, 925 mL. A deep red color indicated a positive reaction. Amino acid decarboxylase activities were determined using a modification of the urease test described by Smibert and Krieg (1981), in which the appropriate amino acid was substituted for urea. For the detection of DNase activity, FBP agar was supplemented with DNA (0.2%) and methyl green (0.005%) (Smith et al. 1969). A loopful of growth from a 24- to 48-h-old culture grown in semisolid Brucella medium was used as the inoculum. A colorless zone with >3 mm total zone size around the area of growth after 72 h was considered a positive reaction.

A modification of a minimal, chemically defined medium of Smibert (1963) was used for growth studies. Minimal medium (MM) contained (g/L): glutamic acid, 2.0; proline, 1.2; aspartic acid, 0.7; leucine, 0.7; niacin, 0.01; $(NH_4)_2SO_4$, 3.0; K_2HPO_4, 4.0; NaCl, 0.05; $Na_2S_2O_3.5H_2O$, 0.8; Na_2CO_3, 0.4; $MgSO_4.7H_2O$, 0.24; $FeSO_4.7H_2O$, 0.012; and agar, 1.6. The pH of MM was adjusted to 7.0 with KOH pellets. Semisolid Brucella medium cultures (24- to 48-h-old) were used as the inoculum: 2 drops from a Pasteur pipet were used to inoculate 5 mL of MM in a 16 x 125-mm tube. Cultures were examined for growth at 24, 48 and 72 h. Semisolid Brucella medium was used as a positive control. A pellicle of growth similar to that occurring in semisolid Brucella medium was considered a positive reaction. A thin haze or no growth was considered negative. In questionable cases, a serial transfer was made to

fresh MM; only those strains showing growth after transfer were considered positive.

The ability to grow aerobically was tested by streaking cultures onto plates of Brucella agar and incubating at 30 or 37°C under an air atmosphere. Aerotolerance was indicated by the occurrence of colonies on all quadrants.

Results

DNA homology results

The 84 strains of catalase-positive campylobacters used in this study could be assigned to seven groups based on the results of DNA homology experiments (Table 2). Group I strains showed 75-100% DNA homology with the type strain of *C. fetus* ssp. *fetus* (ATCC 27374) and 68-96% DNA homology with the type strain of *C. fetus* ssp. *venerealis* (ATCC 19438). The three strains in Group II showed 90-100% DNA homology with the suggested type strain of *"C. hyointestinalis"* (ATCC 35217). Group I and II strains demonstrated 16-26% intergroup DNA homology, but neither group showed significant DNA homology with any of the other catalase-positive campylobacters. The strains comprising Group III all showed high homology (63-100%) with the type strain of *C. jejuni* (ATCC 33560). Group IV strains showed 64-100% homology with the type strain of *C. coli* (ATCC 33559). Group III and IV strains showed 25-49% intergroup homology, but neither showed any significant intergroup homology with the other catalase-positive campylobacters. Group V was made up of three strains having 92-96% DNA homology with strain C729 as the reference strain of *"C. laridis"*. The proposed type strain of *"C. laridis"*, NCTC 11352, showed 96% homology with the reference strain. The five strains in Group VI showed 68-100% homology with strain 14479 as the reference strain of *"C. fecalis"*. The suggested type strain of *"C. fecalis"*, ATCC 33709, showed 100% homology with the reference strain. Group VII consisted of three strains of aerotolerant campylobacters isolated from bovine and porcine abortions, showing 88-100%

DNA homology with reference strain 02790. Groups V, VI and VII showed no significant intergroup DNA homology with any of the other catalase positive campylobacters. Mol% G + C and (T_m) values of the DNA for representative strains are given in Table 3. Mol% G + C values ranged from 29 to 35 for the campylobacters tested.

Biochemical characteristics

The biochemical characteristics of the catalase-positive campylobacters used in this study are summarized in Table 4. All except C. fetus ssp. fetus strain PB 1/1 reduced nitrate and all except the aerotolerant campylobacter strain 02790 grew in the presence of 1% bile. Only "C. fecalis" strains were positive for nitrite reduction, growth in 3.5% NaCl and H_2S production in SIM medium.

Biochemical tests additional to those listed in Table 4 were performed on selected strains of campylobacters representing all of the homology groups. All 11 strains tested for glycine, arginine, leucine, ornithine, lysine, serine, glutamic acid, glutamine and alanine decarboxylase activities and for tolerance to brilliant green were negative. Aspartic acid decarboxylase activity and metronidazole sensitivity varied among these strains, but there was no correlation with homology groups. These and additional strains were tested for aminopeptidase activities. All 66 strains tested for arginyl aminopeptidase were positive. All 42 strains tested for prolyl and alanyl peptidase and all 29 strains tested for cysteinyl aminopeptidase were negative.

Table 5 lists biochemical characteristics useful for differentiating the catalase-positive campylobacters.

Discussion

DNA homology results determined in this study are comparable to those of Belland and Trust (1982), Harvey and Greenwood (1983), Hébert et al. (1983), Leaper and Owen (1982), Owen and Leaper (1981), Owen (1983) and Ursing et al. (1983). Conclusions similar to those based on DNA homology results have been made by Morris and Park (1973) and by Hanna et al. (1983) using electrophoretic patterns of acid-phenol extracts of these organisms.

Our DNA homology results indicate that there are at least seven distinct species of catalase-positive campylobacters. C. jejuni and C. coli have a high degree of interspecies relatedness (25-49% DNA homology), but are separate species, and neither is closely related by DNA homology to any of the other catalase-positive campylobacters. Similar results for these two species have been found by other investigators (Belland and Trust 1982; Harvey and Greenwood 1983; Hébert et al. 1983) and slightly higher (Ursing et al. 1983) and lower (Owen and Leaper 1982; Owen 1983) values have also been reported. Hybridization experiments performed under "stringent" conditions have given lower homology values and thermal stability experiments indicate that there is considerable base-pair mismatching between DNA hybrids from these two species (Belland and Trust 1982; Ursing et al. 1983). Our results indicate that "C. hyointestinalis" is more closely related to C. fetus than to any of the other catalase-positive campylobacters. Mol % G + C values for the campylobacters tested here (Table 3) agree with those reported by others (Belland and Trust 1982;

Leaper and Owen 1982; Owen and Leaper 1981; Owen 1983; Ursing et al. 1983; Véron and Chatelain 1973).

Sensitivity to nalidixic acid has been reported for some C. fetus strains (Harvey and Greenwood 1983; Skirrow and Benjamin (1980) but was not observed in this study. Negative reactions for alkaline phosphatase, DNase, and hippurate hydrolysis have been reported previously for C. fetus (Harvey and Greenwood 1983; Hébert et al. 1982; Smibert 1974), and our results are in agreement. Growth at 25°C was a consistent characteristic for the C. fetus strains tested, but failure to grow at 42°C was not. One strain in particular, Yale, grew equally well at both temperatures, while several other strains showed scanty growth. The ability of some C. fetus strains to grow at 42°C has been reported previously by Smibert and von Graevenitz (1980), Harvey and Greenwood (1983), Skirrow and Benjamin (1980), and Firehammer and Berg (1965). Smibert and von Graevenitz have recommended that strains growing at both 25 and 42°C be identified as C. fetus, and our study supports this recommendation.

Strains of C. fetus ssp. fetus and C. fetus ssp. venerealis cannot be distinguished from one another by DNA homology experiments. Growth in 1% glycine is presently the only laboratory test that can be used to separate ssp. fetus from ssp. venerealis, and the strains used in our study have been separated on that basis. Chang and Ogg (1971) reported that tolerance to 1% glycine can be obtained through transduction or mutation. For these reasons, it has been suggested that there is no justification for retaining the subspecies designations for C. fetus (Harvey and Greenwood 1983; Hébert et al. 1983). However, acquisition of glycine tolerance occurs in a stepwise

fashion (Chang and Ogg 1971) and this would seem unlikely to occur readily. In our laboratory strains of C. fetus ssp. venerealis have never become glycine tolerant, even though some have been maintained for up to 20 years. Moreover, there are two very practical reasons for maintaining these taxa. First, C. fetus ssp. venerealis is associated with a form of contagious abortion in cattle that is transmitted venereally and this organism cannot survive in the bovine intestinal tract. On the other hand, C. fetus ssp. fetus, which can survive in the intestinal tract of both cattle and sheep, causes a sporadic abortion in both of these animals that is spread by ingestion of contaminated materials (Bryner 1964; 1971). In treating a herd beset with fertility problems associated with C. fetus, the veterinarian would benefit greatly by knowing whether the causative agent was being transmitted venereally or by ingestion. Second, C. fetus ssp. venerealis rarely, if ever, causes human disease (Rettig 1979), whereas C. fetus ssp. fetus is a potentially dangerous human pathogen which can cause severe blood infections in individuals with compromised immune systems (Rettig 1979). One strain of C. fetus ssp. fetus used in this study (ATCC 33561) was received under the name C. fetus ssp. venerealis biotype intermedius and was isolated from human blood. However, this organism grew in 1% glycine and apparently was misnamed. Hébert et al. (1983) reported similar results for a C. fetus ssp. venerealis biotype intermedius strain.

"C. hyointestinalis" has been isolated from pigs with proliferative ileitis and shows a closer relationship with C. fetus by DNA homology than with any of the other catalase-positive campylobacters. "C. hyointestinalis" strains resembled some C. fetus ssp. fetus strains in being able to grow at

both 25 and 42°C and to grow in the presence of 1% glycine, and in being resistant to nalidixic acid but sensitive to cephalothin. The three strains of "C. hyointestinalis" used in this study produced small amounts of H_2S on TSI slants and were able to grow anaerobically in 0.1% TMAO, while none of the C. fetus strains produced H_2S on TSI slants or grew anaerobically in TMAO. Goodman and Hoffman (1983) have reported that a strain of "C. hyointestinalis" (80-4577-4) is positive for hydrogenase activity and that C. fetus is negative. This may be another useful characteristic for separating these two species.

C. coli and C. jejuni strains were very similar in their biochemical characteristics and easily differentiated from C. fetus by their inability to grow at 25°C, resistance to cephalothin, and sensitivity to nalidixic acid. One C. jejuni strain (PC340) and two C. coli strains (541 and 80-1184-2) were sensitive to cephalothin, but the others were resistant. Harvey and Greenwood (1983) reported that all of their C. coli and C. jejuni strains were resistant to cephalothin. C. coli (75%) and C. jejuni (95%) were the only catalase-positive campylobacters that showed alkaline phosphatase activity. A higher proportion of C. jejuni strains were found to be positive in this study than has been previously reported (Hébert et al. 1982; Smibert 1974). This discrepancy may be due to the fact that in the previous reports data on C. jejuni and C. coli strains were combined because these two taxa were considered as a single species. In our study, forty percent of the C. jejuni strains and 67% of the C. coli strains were positive for DNase activity. If these results are combined, 48% of the (C. coli/C. jejuni) strains were DNase positive, which is very similar to the values obtained by Hébert et al.

(1982) and Smibert (1974) for (C. coli/C. jejuni). DNase and alkaline phosphatase are not useful characters for differentiating C. coli from C. jejuni, but may be useful when combined with other tests for biotyping C. coli and C. jejuni strains. Indeed, Hébert et al. (1982) have proposed such a biotyping scheme.

Several phenotypic tests have been proposed to differentiate C. coli from C. jejuni, but to date the only reliable one has been rapid hippurate hydrolysis (Harvey 1980; Harvey and Greenwood 1983; Leaper and Owen 1981). All C. coli strains used in our study were negative for hippurate hydrolysis whereas 20 of 21 C. jejuni strains hydrolyzed hippurate. In addition, 11 of 12 C. coli strains were able to grow in a semisolid minimal medium (MM), while none of the 20 C. jejuni strains tested were able to grow in this medium. Eleven of 12 C. coli strains produced a band of H_2S at the junction of the slant and butt of a TSI slant provided that sufficient water of syneresis was present. No C. jejuni strains produced H_2S on TSI slants.

C. jejuni is an important cause of gastroenteritis in man (Butzler et al. 1973; Skirrow 1977). The frequency with which this organism is isolated from stool samples has increased greatly in recent years due to the development of specific selective media and the recognition that C. jejuni requires microaerobic growth conditions. C. coli has been reported from cases of human diarrhea (Leaper and Owen 1981; this study) but does not appear to be nearly as important in this respect as C. jejuni. Luechtefeld and Wang (1982) reported that 99% of the campylobacters they isolated from diarrheic stools hydrolyzed hippurate. This indicates that C. jejuni is the predominant

cause of campylobacter-associated gastroenteritis. C. jejuni also causes abortion in sheep and diarrhea in young animals (Smibert 1978) and can be isolated from healthy cattle, sheep, zoo animals, birds and poultry, while C. coli appears to be restricted to hogs and poultry (Luechtefeld and Wang 1982). Since infections due to these two organisms are probably transmitted by ingestion of contaminated food and water, it would appear that the animals that normally harbor C. jejuni would be a much more likely source of human infection than those that harbor C. coli. Thus in ecological or epidemiological studies, it is important to accurately identify both species.

"C. laridis" is the name recently proposed by Benjamin et al. (1983) to describe those organisms formerly referred to as NARTC (nalidixic acid-resistant thermophilic Campylobacter). These strains were originally isolated from wild seagulls, but have also been isolated from humans, cattle, dogs, ducks, a foal, a seal and a monkey (Benjamin et al. 1983). The proposed type strain of "C. laridis", NCTC 11352, was not available when this study began and strain C729 was used as a reference strain; however, strain C729 has 96% DNA homology with NCTC strain 11352. The DNA homology results reported here are similar to those of Benjamin et al. (1983), Harvey and Greenwood (1983), Leaper and Owen (1982), Owen and Leaper (1981), and Ursing et al. (1983), who reported that "C. laridis" has very little DNA homology with any of the other catalase-positive campylobacters.

"C. laridis" strains were similar to C. jejuni and C. coli strains in their inability to grow at 25°C and their resistance to cephalothin and, like C. coli, they did not hydrolyze hippurate. However, they differed from both C. coli and C. jejuni strains in their resistance to nalidixic acid. They were also

negative for alkaline phosphatase and DNase activity. The ability of "C. laridis" to grow anaerobically in 0.1% trimethylamime-N-oxide (TMAO) and to grow in the presence of 1.5% NaCl have been proposed as additional means for distinguishing this species from C. coli and C. jejuni (Benjamin et al. 1983). This is supported by our results.

To date, "C. laridis" has not been reported as a cause of human disease. However, two of the "C. laridis" strains used in this study were isolated from clinical samples, suggesting that "C. laridis" may be a human pathogen.

"C. fecalis is a saprophyte isolated from sheep feces (Firehammer 1965). It is easily distinguished from the other catalase-positive campylobacters by its salt tolerance (3.5% NaCl) and abundant H_2S production. These characteristics are usually associated with the catalase-negative campylobacters. It is possible that "C. fecalis" is a variant of a catalase-negative campylobacter that has acquired catalase activity (Harvey and Greenwood 1983).

The aerotolerant campylobacters described by Neill et al. (1979) were isolated from bovine and porcine abortions. These organisms have many characteristics in common with the catalase-positive campylobacters, but they grow aerobically. Three strains used in this study were highly related to each other (88-100% DNA homology), but did not show significant DNA homology with any of the other reference strains used. Recently, Hanna et al. (1983) demonstrated that these organisms showed different electrophoretic protein profiles than did reference strains of the recognized Campylobacter species. In addition, they reported that these organisms had

been isolated from ovine and equine abortions, as well as bovine and porcine abortions.

The aerotolerant campylobacter strains used in this study showed phenotypic characteristics similar to C. fetus ssp. venerealis (e.g., growth at 25°C, but not at 42°C, and sensitivity to 1% glycine) with two notable exceptions: C. fetus ssp. venerealis could not grow aerobically and was sensitive to cephalothin, while the aerotolerant campylobacters were resistant to cephalothin. These organisms have not been implicated in human infection.

The majority of the tests described in this report have long been used to differentiate the members of the genus Campylobacter into their respective species and subspecies. The reliability of these tests was examined by correlating species assignment by DNA homology groups with biochemical and physiologic tests. The biochemical and physiological characteristics that were found to be the most reliable for identifying the catalase-positive campylobacters are shown in Table 5. These findings should prove useful not only for identifying newly isolated strains but also for epidemiological purposes.

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TABLE 1. Bacterial strains used in this study.

Species and Strain	Source	Origin
C. fetus ssp. *fetus*:		
ATCC 27374	American Type Culture Collection (type strain of *C. fetus* subspecies *fetus*)	Ovine: brain
DT74[a]	B. D. Firehammer, Veterinary Research Laboratory, Montana State University, Bozeman	Bovine
Yale	A. von Graevenitz, Yale University School of Medicine, New Haven, Connecticut	Human: blood
0053B76	California Department of Health	Human: blood
19306	M. J. R. Gilchrist, Mayo Clinic, Rochester, Minnesota	Human: blood
11324A76	California Department of Health	Human: blood
875B	California Department of Health	Human: feces
HF1	Henry Ford Hospital, Detroit, Michigan	Human: blood
11639B76	UCLA Hospital, Los Angeles, California	Human
V8	J. Harkness, St. Vincent's Hospital, NSW, Australia	Human: blood
436	J. Bryner, Animal Research Service, Ames, Iowa	Bovine: fetus
Langford[b]	B. D. Firehammer	Ovine

TABLE 1, continued.....

Species and Strain	Source	Origin
10583	J. Smith, Indiana University School of Medicine, Indianapolis, Indiana	Human: blood
PB 1/1	V. Bokkenheuser, St. Luke's Hospital, New York City	Human: blood
937	J. A. Kellogg, York Hospital, York, Pennsylvania	Human: blood
775-A-78	California Department of Health	Human: blood
4099	G. Demko, Peninsula General Hospital, Salisbury, Maryland	Human: blood
Pacemaker	P. Kunkle, University of Nebraska Medical Center, Omaha, Nebraska	Human: pacemaker fluid
11529	W. A. Jackson, Bureau of Bacteriology, LCDC, Ottawa	Human: blood
1510MB	P. Yam, University of Nebraska Medical Omaha, Nebraska	Human: blood
Suis VI	J. Bryans, University of Kentucky, Lexington, Kentucky	Porcine
ATCC 33247	American Type Culture Collection	Human: blood
B6286	R. E. Weaver, Centers for Disease Control, Atlanta, Georgia	Human
Suis I	J. Bryans	Porcine

TABLE 1, continued.....

Species and Strain	Source	Origin
273[c]	B. D. Firehammer	Bovine
HCB[d]	B. D. Firehammer	Bovine
ATCC 33561	American Type Culture Collection	Human: blood
1908B79	California Department of Health	Human: blood
Wang I	W.-L. L. Wang, Denver Veterans Administration Medical Center, Colorado	Human: blood
UCLA 80997	UCLA Hospital, Los Angeles, California	Human: blood
Wang II	W.-L. L. Wang	Human: blood
12244	Florida Department of Health	Human: blood
C. fetus ssp. *venerealis*:		
ATCC 19438	American Type Culture Collection (type of *C. fetus* subspecies *venerealis*	Bovine: vaginal mucus
7721	Florida Department of Agriculture	Bovine: fetus
998[e]	J. Bryner	Bovine: vaginal mucus
B24MK	J. Bryans	Bovine
UMHS	J. Bryans	Bovine

TABLE 1, continued.....

Species and Strain	Source	Origin
"C. hyointestinalis":		
80-4577-4[f] (ATCC 35217)	C. Gebhart, College of Veterinary Medicine, University of Minnesota, Minneapolis, Minnesota	Porcine: feces
81-13087	C. Gebhart	Porcine: feces
81-14151-1	C. Gebhart	Porcine: feces
C. jejuni:		
ATCC 33560	American Type Culture Collection (type strain of C. jejuni)	Bovine: feces
B7619	R. E. Weaver	Human: feces
H325	P. Dekeyser, St. Pierre Hospital, Free University of Brussels, Belgium	Human: feces
H840	P. Dekeyser	Human: feces
4164A	California Department of Health	Human: feces
12019	Florida Department of Health	Human: blood
12375	California Department of Health	Human: blood
13136[g]	B. D. Firehammer	Ovine: fetus
82214	UCLA Hospital, Los Angeles	Human: cord blood

TABLE 1, continued.....

Species and Strain	Source	Origin
Veit	College of Veterinary Medicine, Virginia Polytechnic Institute, Blacksburg	Ovine: fetus
Holy Cross	J. Benonati, Holy Cross Hospital, Fort Lauderdale, Florida	Human: blood
8916[h]	B. D. Firehammer	Ovine: bile
ATCC 33250	American Type Culture Collection	Human: blood
ATCC 33251	American Type Culture Collection	Human: blood
N82-11A	College of Veterinary Medicine, Virginia Polytechnic Institute, Blacksburg	Ovine: fetus
11151	California Department of Health	Human: blood
61-784	J. Bryans	Chicken
12191	California Department of Health	Human: blood
N82-10A	College of Veterinary Medicine, Virginia Polytechnic Institute, Blacksburg	Ovine: fetus
11641B76	California Department of Health	Human: blood
PC340	J. L. Penner, Banting Institute, University of Toronto	Unknown

TABLE 1, continued.....

Species and Strain	Source	Origin
C. coli:		
ATCC 33559	American Type Culture Collection (type strain of *C. coli*)	Porcine: feces
80-15573	G. E. Ward, College of Veterinary Medicine, University of Minnesota, St. Paul	Porcine: feces
81-3592-3	G. E. Ward	Porcine: feces
7292B76	California Department of Health	Human: blood
H550	P. Dekeyser	Human: feces
C699	H. Lior, National Enteric Reference Center, LCDC, Ottawa	Chicken: feces
C607	H. Lior	Porcine: feces
C602	H. Lior	Porcine: feces
C679	H. Lior	Chicken: feces
541	R. M. Smibert, Virginia Polytechnic Institute, Blacksburg, Virginia	Porcine: feces
76-3227	D. Lamb, Emory University, Atlanta, Georgia	Human: blood
80-1184-2	G. E. Ward	Porcine: feces

TABLE I, continued.....

Species and Strain	Source	Origin
"C. laridis"		
C729	H. Lior	Human: feces
1789	I. Nachamkin, University of Pennsylvania School of Medicine, Philadelphia	Human: blood
NCTC 11352	National Collection of Type Cultures, London, (proposed type strain of "C. laridis"	Avian: feces
"C. fecalis"		
14479	B. D. Firehammer	Ovine: feces
14223	B. D. Firehammer	Ovine: feces
11362	B. D. Firehammer	Ovine: feces
11411	B. D. Firehammer	Ovine: feces
ATCC 33709[1]	B. D. Firehammer	Ovine: feces
Aerotolerant campylobacters		
02790	S. D. Neill, Veterinary Research Laboratory, Belfast, Ireland	Porcine: fetus
02776	S. D. Neill	Porcine: fetus
02824	S. D. Neill	Bovine: fetus

TABLE 1, continued.....

[a]Berg et al. (1971) serovar B, heat-labile antigens 2, 4, 6, 7; Marsh and Firehammer (1953) serovar II-III; Morgan (1959) serovar B.

[b]Berg et al. (1971) serovar B, heat-labile antigens 2, 5; Morgan (1959) serovar B; Marsh and Firehammer (1953) serovar II-V; Mitscherlich and Liess (1958) serovar 2.

[c]Berg et al. (1971) serovar B, heat-labile antigen 2; Marsh and Firehammer (1953) serovar II; Mitscherlich and Liess (1958) serovar 2.

[d]Berg at al. (1971) serovar A_1; Marsh and Firehammer (1953) serovar III-II, heat-labile antigens 3, 4.

[e]Berg et al. (1971) serovar A-2, heat-labile antigens 3, 6; Morgan (1959) serovar A; Marsh and Firehammer (1953) serovar III.

[f]Suggested type strain of "C. hyointestinalis", deposited with the American Type Culture Collection (C. Gebhart, personal communication).

[g]Berg et al. (1971) serovar C; Marsh and Firehammer (1953) serovar I; Mitscherlich and Liess (1958) serovar 13.

[h]Berg et al. (1971) serovar C; Marsh and Firehammer (1953) serovar I.

[i]Suggested type strain of "C. fecalis", deposited with the American Type Culture Collection (B. D. Firehammer, personal communication).

TABLE 2. DNA homology groups of catalase-positive campylobacters.

Unlabeled DNA from strain	% Homology to DNA of reference strains							
	C. fetus subsp. *fetus* ATCC 27374	*C. fetus* subsp. *venerealis* ATCC 19438	"*C. hyointestinalis*" 80-4577-4	*C. jejuni* ATCC 33560	*C. coli* ATCC 33559	"*C. laridis*" C729	"*C. fecalis*" 14479	Aerotolerant campylobacter 02790
GROUP I (*C. fetus*)								
C. fetus subsp. *fetus*								
ATCC 27374	100	85	26	3	16	5	4	3
DT74	100	80	ND[a]	8	ND	ND	ND	ND
Yale	97	90	25	7	4	4	7	0
0053B76	96	89	ND	6	4	5	ND	0
19306	96	86	23	16	12	ND	4	0
11324A76	96	90	ND	3	4	ND	ND	0
875B	94	85	23	13	7	ND	18	0
HF1	94	79	25	5	5	ND	20	0
11639B	92	77	ND	15	12	ND	ND	5
V8	92	91	23	5	5	4	15	5
436	92	91	ND	5	4	ND	ND	3
Langford	92	79	ND	5	6	ND	ND	1
10583	92	82	27	6	4	4	ND	3
PB 1/1	91	85	25	8	4	ND	21	7
937	91	76	ND	7	6	ND	ND	ND
775-A-78	91	87	25	3	4	7	ND	0
4099	90	82	24	14	5	ND	17	4
Pacemaker	90	84	24	6	5	6	22	0
11529	89	85	ND	5	3	ND	ND	ND

TABLE 2, continued.....

Unlabeled DNA from strain	% Homology to DNA of reference strains							
	C. fetus subsp. *fetus* ATCC 27374	*C. fetus* subsp. *venerealis* ATCC 19438	"*C. hyointestinalis*" 80-4577-4	*C. jejuni* ATCC 33560	*C. coli* ATCC 33559	"*C. laridis*" C729	"*C. fecalis*" 14479	Aerotolerant campylobacter 02790
1510MB	89	81	23	17	3	5	16	1
Suis VI	89	79	ND	12	3	4	ND	6
ATCC 33247	89	68	ND	4	5	ND	ND	ND
B6286	88	96	ND	16	4	ND	ND	ND
Suis I	88	81	23	2	4	ND	ND	7
273	88	78	ND	43	13	ND	ND	ND
HCB	87	85	ND	23	6	ND	ND	ND
ATCC 33561	86	91	ND	5	5	2	9	6
1908B79	85	85	ND	11	4	ND	ND	15
Wang I	84	85	ND	14	ND	ND	ND	ND
UCLA 80997	80	78	ND	15	2	ND	ND	8
Wang II	78	76	ND	14	ND	ND	ND	ND
12244	75	80	16	18	5	ND	10	10
C. fetus subsp. *venerealis* ATCC 19438	91	100	23	16	4	4	19	ND
UMHS	91	95	25	3	5	5	17	7
B24MK	93	95	26	5	10	ND	19	0
7721	97	90	23	4	4	5	16	3
998	94	84	ND	9	7	ND	ND	5

TABLE 2, continued.....

Unlabeled DNA from strain	% Homology to DNA of reference strains							
	C. fetus subsp. fetus ATCC 27374	C. fetus subsp. venerealis ATCC 19438	"C. hyointestinalis" 80-4577-4	C. jejuni ATCC 33560	C. coli ATCC 33559	"C. laridis" C729	"C. "fecalis" 14479	Aerotolerant campylobacter 02790
GROUP II								
"C. hyointestinalis"								
80-4577-4	24	19	100	8	13	6	12	2
81-14151-1	ND	ND	95	ND	ND	5	16	ND
81-13087	ND	ND	90	2	ND	4	6	ND
GROUP III								
C. jejuni								
ATCC 33560	3	6	2	100	35	10	3	4
B7619	ND	13	ND	89	ND	ND	ND	ND
H325	7	9	4	86	39	14	15	3
H840	4	3	3	86	40	14	11	4
4164A	2	13	ND	86	39	ND	ND	10
12019	5	5	4	85	41	14	18	0
12375	4	6	3	83	38	ND	17	3
13136	7	3	4	82	41	14	11	0
82214	3	5	ND	81	40	15	17	2
Veit	5	3	4	81	39	12	2	5
Holy Cross	3	4	4	80	37	14	17	8
8916	14	10	6	80	40	14	10	0
ATCC 33250	6	6	5	80	36	ND	15	1

TABLE 2, continued.....

Unlabeled DNA from strain	% Homology to DNA of reference strains							
	C. fetus subsp. *fetus* ATCC 27374	*C. fetus* subsp. *venerealis* ATCC 19438	"*C. hyointestinalis*" 80-4577-4	*C. jejuni* ATCC 33560	*C. coli* ATCC 33559	"*C. laridis*" C729	"*C. fecalis*" 14479	Aerotolerant campylobacter 02790
ATCC 33251	2	0	2	80	29	ND	8	0
N82-11A	4	4	3	79	38	ND	21	ND
11151	8	9	5	79	37	14	15	2
61-784	8	9	ND	77	35	ND	ND	3
12191	5	1	3	75	40	ND	5	2
N82-10A	1	0	2	73	34	13	ND	5
11641B76	8	4	3	64	31	8	7	3
PC340	0	0	0	63	25	12	ND	0
GROUP IV								
C. coli								
ATCC 33559	2	2	2	39	100	13	5	ND
80-15773	8	2	5	38	89	14	15	2
81-3592-3	30	26	9	49	89	15	ND	4
7292B76	7	6	5	41	89	ND	17	8
H550	6	5	ND	42	89	13	ND	0
C699	9	7	ND	37	87	15	ND	0
C607	4	3	5	39	86	14	17	0
C602	5	5	4	36	86	15	16	0
C679	11	8	4	40	84	ND	3	3
541	4	1	2	33	79	9	4	1

TABLE 2, continued.....

Unlabeled DNA from strain	% Homology to DNA of reference strains							
	C. fetus subsp. fetus ATCC 27374	C. fetus subsp. venerealis ATCC 19438	"C. hyointestinalis" 80-4577-4	C. jejuni ATCC 33560	C. coli ATCC 33559	"C. laridis" C729	"C. "fecalis" 14479	Aerotolerant campylobacter 02790
76-3227	2	4	4	34	64	13	12	0
80-1184-2[b]	35	30	ND	25	ND	ND	ND	ND
GROUP V "C. laridis"								
C729[c]	13	7	16	16	ND	100	5	3
NCTC 11352	ND	ND	5	ND	ND	96	ND	ND
1789	ND	ND	ND	19	ND	92	ND	ND
GROUP VI "C. fecalis"								
14479	ND	ND	4	1	ND	4	100	ND
11362	ND	ND	12	11	ND	14	100	ND
ATCC 33709	ND	ND	8	11	ND	7	100	ND
14223	ND	ND	6	7	ND	5	96	ND
11411	ND	ND	6	1	ND	19	80	ND

TABLE 2, continued.....

Unlabeled DNA from strain	% Homology to DNA of reference strains							
	C. fetus subsp. *fetus* ATCC 27374	*C. fetus* subsp. *venerealis* ATCC 19438	"*C. hyointestinalis*" 80-4577-4	*C. jejuni* ATCC 33560	*C. coli* ATCC 33559	"*C. laridis*" C729	"*C. fecalis*" 14479	Aerotolerant campylobacter 02790
GROUP VII Aerotolerant campylobacters								
02790	4	2	3	7	6	5	7	100
02776	6	3	ND	3	2	2	ND	94
02824	ND	ND	2	2	ND	5	13	88

[a]ND = not done.

[b]DNA from strain 80-1184-2 was not hybridized with DNA from *C. coli* ATCC 33559, but was used in a preliminary study employing reference DNA from *C. coli* strain 541 and was found to have a DNA homology value of 82% with this strain; consequently, it is related at the species level to Group IV.

[c]DNA from strain C729 was not hybridized with DNA from *C. coli* ATCC 33559, but was used in a preliminary study employing reference DNA from *C. coli* strain 541 and was found to have a DNA homology value of 25% with this strain; consequently, it is not related at the species level to Group IV.

TABLE 3. Mol% G + C values of representative Campylobacter strains.

Species	Strain	T_m (°C)[a]	Mol% G + C[b] (±1%)
C. fetus subsp. fetus	ATCC 27374	81.4	33
	Wang II	82.2	34
	Yale	81.8	33
C. fetus subsp. venerealis	ATCC 19438	81.5	33
	UMHS	81.9	34
	998	82.0	34
"C. hyointestinalis"	80-4577-4	82.4	35
C. jejuni	ATCC 33560	80.1	30
	Veit	81.0	32
	H840	81.0	32
C. coli	ATCC 33559	80.5	31
	541	80.7	32
	C699	81.5	33
"C. laridis"	C729	80.0	30
"C. fecalis"	14223	81.0	32
Aerotolerant campylobacters	02790	79.3	29
	02776	79.9	30

[a]Values normalized for 1 X SSC (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0).

[b]Calculated using the equation of Mandel et al. (1970).

TABLE 4. Physiological and biochemical characteristics of the Campylobacter strains used in this study.

Characteristic	C. fetus subsp. fetus	C. fetus subsp. venerealis	"C. hyointestinalis"	C. jejuni	C. coli	"C. laridis"	"C. fecalis"	Aerotolerant campylobacters
DNA homology group	I	I	II	III	IV	V	VI	VII
No. of strains tested	32	5	3	21	12	3	5	3
Catalase, oxidase	32[a]	5	3	21	12	3	5	3
Indol production, oxidation or fermentation of carbohydrates	0	0	0	0	0	0	0	0
H_2S production:								
SIM medium	0	0	0	0	0	0	5	0
lead acetate strip method	23	1	3	18	12	3	5	0
TSI slant	0	0	3	0	12	0	5	0
Growth in:								
1% glycine	32	0	3	21	12	3	5	0
1% oxgall	32	5	3	21	12	3	5	2
1.5% NaCl (plates)	5	0	0	0	0	3	0	0
3.5% NaCl	0	0	0	0[f]	0	0	5	0
minimal medium	11	1	0	0	11	0	1	1

TABLE 4, continued.....

Characteristic	*C. fetus* subsp. *fetus*	*C. fetus* subsp. *venerealis*	"*C. hyointestinalis*"	*C. jejuni*	*C. coli*	"*C. laridis*"	"*C. fecalis*"	Aerotolerant campylobacters
Anaerobic growth in 0.1% TMAO	0	0	3	0	1	3	5	0
Growth at:								
25°C	32	5	3	0	0	0	0	3
42°C	4	0	3	21	12	3	5	0
Hippurate hydrolysis	0	0	0	20	0	0	0	0
Sensitive to:								
nalidixic acid[b]	0	0	0	21[d]	11[e]	0	0	1
cephalothin[c]	32	5	3	1[d]	2[e]	0	5	0
Alkaline phosphatase	0	0	0	19[f]	9	0	0	0
DNase	0	0	0	8[f]	8	0	5	0
Nitrate reduced to nitrite	31	5	3	21	12	3	5	3
Nitrite reduction	0	0	0	0	0	0	5	0

TABLE 4, continued.....

[a]Values indicated are the number of strains giving a positive reaction.

[b]30 µg disks were used. Zone diameters of sensitive strains ranged from 17-32 mm.

[c]30 µg disks were used. Zone diameters of sensitive strains ranged from 10-47 mm.

[d]C. jejuni strain PC340 was sensitive to cephalothin, showing a zone diameter of 13 mm (18 mm secondary zone).

[e]C. coli strains 80-1184-2 and 541 were sensitive to cephalothin, showing zone diameters of 25 and 30 mm, respectively.

[f]Only 20 strains of C. jejuni were tested for alkaline phosphatase activity, DNase activity, and the ability to grow in minimal medium. Strain 8916 died and hence could not be tested.

TABLE 5. Differential characteristics of the catalase-positive campylobacters.[a]

Characteristic	*C. fetus* subsp. *fetus*	*C. fetus* subsp. *venerealis*	"*C. hyointestinalis*"	*C. jejuni*	*C. coli*	"*C. laridis*"	"*C. fecalis*"	Aerotolerant campylobacters
Growth at:								
25°C	+	+	+	-	-	-	-	+
42°C	[-]	-	+	+	+	+	+	-
Growth in:								
1% glycine	+	-	+	+	+	+	+	-
minimal medium	d	[-]	-	-	+	-	[-]	d
1.5% NaCl (plates)	[-]	-	-	-	-	+	-	-
3.5% NaCl	-	-	-	-	-	-	+	-
Anaerobic growth in 0.1% TMAO	-	-	+	-	-	+	+	-
Sensitive to:								
nalidixic acid, 30 µg disk	-	-	-	+	+	-	-	d
cephalothin, 30 µg disk	+	+	+	-	[-]	-	+	-
Hippurate hydrolysis	-	-	-	+	-	-	-	-
H_2S production:								
SIM medium	-	-	-	-	-	-	+	-
TSI medium	-	-	+	-	+	-	+	-

TABLE 5, continued.....

Characteristic	*C. fetus* subsp. *fetus*	*C. fetus* subsp. *venerealis*	"*C. hyointestinalis*"	*C. jejuni*	*C. coli*	"*C. laridis*"	"*C. fecalis*"	Aerotolerant campylobacters
Alkaline phosphatase	-	-	-	+	d	-	-	-
Aerobic growth	-	-	-	-	-	-	-	+
Nitrite reduction	-	-	-	-	-	-	+	-

[a]Symbols: + = 90-100% of strains positive; d = 26-75% of strains positive; [-] = 11-25% of strains positive; - = 0-10% of strains positive.

Chapter 2

Improved Biotyping Schemes for

Campylobacter jejuni and *Campylobacter coli*

Abstract

Camplyobacter jejuni (20 strains) and Campylobacter coli (12 strains) were assigned to four biovars for each species based on phenotypic tests that were easy to perform and interpret. The resulting biotyping schemes offer a greater degree of distinction among C. jejuni and C. coli strains than any of the other biotyping schemes previously described for these organisms.

Campylobacter jejuni is a major cause of gastroenteritis in humans (4, 20) and is currently being isolated as frequently as Salmonella and Shigella species (2, 3). C. coli also produces human enteric disease, but is not encountered as frequently as C. jejuni (14, 22). Although C. jejuni and C. coli have many phenotypic characteristics in common, DNA homology studies have shown that they are separate species (1, 8, 10, 12, 15, 16, 19, 25). The host range for these two organisms is also different: C. coli is isolated primarily from pigs and poultry (14) and occasionally from dogs (19), whereas C. jejuni can be isolated from cattle, sheep, dogs, cats, poultry and other animals (5, 14). For this reason, accurate identification to the species level is necessary for studying the epidemiology of campylobacter-associated gastroenteritis. Identification of a strain as belonging to a particular biovar (biotype) or serotype is also very useful in epidemiological studies. There are several serotyping schemes that have been developed for these organisms (10, 13, 17) and they have proven very useful, but the reagents for these schemes are not commercially available and a recent report indicates that speciation by serotyping is not always accurate (11).

To date, three schemes have been proposed for biotyping C. jejuni and C. coli strains (9, 21, H. Lior, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C279, p. 358). One is useful only for C. jejuni (21) and another does not differentiate between C. jejuni and C. coli strains (9). Lior's scheme can be applied to C. jejuni or C. coli strains. This report describes new schemes for biotyping both C. jejuni and C. coli strains that are simple to use and easy to interpret.

The campylobacter strains used in this study were isolated from a variety of animals (Table 1) and were identified to the species level by both their phenotypic characteristics and high DNA homology (>60%) with their type strains (18).

Alkaline phosphatase activity was measured using a modification of the technique described by Hébert et. al. (9). The top 1-2 ml of growth from two cultures grown in semisolid Brucella medium for 48 h was used to inoculate a Roux bottle containing 200 ml of Brucella agar supplemented with 0.025% each ferrous sulfate, sodium bisulfite and sodium pyruvate (FBP agar) (6). The agar was overlayed with 50 ml of Brucella broth supplemented with 0.5% ferrous sulfate and sodium pyruvate and 0.25% sodium bisulfite (FBP broth) (6). Roux bottles were incubated aerobically at 37°C for 24 h. Cells were harvested at 12,000 x g in a refrigerated centrifuge and washed once with 0.85% sterile saline. The cells were suspended in 0.85% saline and adjusted to a turbidity equivalent to a #2 McFarland standard. One-half ml of the cell suspension was dispensed into a 16 x 125 mm test tube and 0.5 ml of alkaline phosphatase reagent (0.2% p-nitrophenylphosphate dissolved in 0.005M glycine, 5 x 10^{-4} M $MgCl_2$, pH 10.5) (9) was added. Tubes were incubated for 6 h at 37°C. Any yellow color was considered a positive reaction. C. jejuni strain 13136 served as a positive control and sterile saline was the negative control. FBP agar plates supplemented with 0.2% DNA and 0.005% methyl green (24) were used to detect DNase activity. Growth from a 48-h-old semisolid culture was used as the inoculum and plates were incubated at 37°C under an atmosphere of 6% O_2, 5% CO_2 and 89% N_2 for 72 h. A zone of clearing greater than 3 mm around the area of growth was

considered a positive reaction. Hippurate hydrolysis was determined using the method of Harvey (7). The ability of strains to grow in a minimal medium was tested in a semisolid medium (MM) composed of (g/l): glutamic acid, 2.0; proline, 1.2; aspartic acid, 0.7; leucine, 0.7; niacin. 0.01; $(NH_4)_2SO_4$, 3.0; K_2HPO_4, 4.0; NaCl, 0.05; $Na_2S_2O_3.5H_2O$, 0.8; Na_2CO_3, 0.4; $MgSO_4.7H_2O$, 0.24; $FeSO_4.7H_2O$, 0.012; and agar, 1.6. The pH was adjusted to 7.0 with KOH. MM is a modification of a medium described previously by Smibert (23). Two drops from a Pasteur pipet of a culture grown in semisolid Brucella medium for 24 to 48 h was used to inoculate 5 ml of MM in a 16 x 125 mm tube. Cultures were examined for growth at 24, 48 and 72 h. Semisolid Brucella medium was used as a positive control. A pellicle of growth similar to that occurring in semisolid Brucella medium was considered a positive reaction. A thin haze or no growth was considered negative. In questionable cases, a serial transfer was made to fresh MM; only those strains showing growth after transfer were considered positive. All of the above tests were done in triplicate. Any test yielding questionable results was repeated.

The 20 C. jejuni strains used in this study could be assigned to 4 biovars based on alkaline phosphatase and DNase activity and hippurate hydrolysis (Table 1). Hippurate hydrolysis is an important characteristic for identifying C. jejuni, but an occasional strain will be hippurate negative (8, 11, 18). Other characteristics can be used to differentiate C. jejuni from C. coli (Table 2). It is important to properly identify these organisms to the species level before assigning biovar designations. Seventy-five percent of the human strains used in this study belonged to biovar 2, which was also represented by a bovine and an ovine strain. It would be interesting to

determine if this is the predominant biovar among strains of C. jejuni isolated from poultry, because poultry have been suggested as an important source of human campylobacter infections (22). Unfortunately, only one strain of C. jejuni from poultry was available for use in this study, and it belonged to biovar 1.

Alkaline phosphatase and DNase activity and the ability to grow in a minimal medium (MM) were used to place 12 C. coli strains into 4 biovars (Table 1). An occasional strain of C. coli may fail to grow in MM, and this can be a useful trait for biotyping if the strain has been properly identified to the species level. All 3 human isolates used here belonged to biovar 1, which also included 4 pig isolates and a poultry isolate. Pork has been suggested as a possible source of human infection in several European countries, and in these countries there is a higher rate of isolation of C. coli from human illness than there is from Britain or the United States (14, 22).

The biotyping scheme proposed by Skirrow and Benjamin (21) is useful only for C. jejuni strains and is based on the production of H_2S in an iron-based "sensitive" medium. All of the 20 strains used in this study were Skirrow biotype 1. The schemes proposed in this report separate C. jejuni into more biovars than does that of Skirrow and Benjamin and also provide a scheme that is useful for C. coli. Even though C. coli is not as prevalent in human disease as C. jejuni, it is a human pathogen and epidemiological markers for C. coli would prove very useful in an outbreak of gastroenteritis caused by C. coli.

Lior (Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C279, p. 358) developed a biotyping scheme based on rapid H_2S production, DNase activity

and hippurate hydrolysis that is basically an expansion of that proposed by Skirrow and Benjamin (21). This scheme is useful for both C. jejuni and C. coli, but the number of potential biotypes for each species is limited to 4 for C. jejuni and 2 for C. coli. However, the schemes described in this report could be expanded to 8 potential biovars for each species.

Hébert et al. (10) proposed a biotyping scheme for C. jejuni and C. coli strains based on DNase activity, hippurate hydrolysis and growth on charcoal-yeast extract (CYE) agar and have applied it to 123 human isolates. One problem with this scheme is that C. jejuni and C. coli strains are not differentiated. A biotyping scheme is most useful if the strains are properly identified to the species level before they are assigned to biovars. Another problem with this scheme is the use of CYE agar which is somewhat difficult to make; moreover, we found it difficult to determine growth responses on CYE agar. In contrast, the minimal medium used in the biotyping scheme described in this report for C. coli is easy to prepare and the growth reactions are very simple to interpret.

C. jejuni and to a lesser extent, C. coli are important causes of gastroenteritis in humans. The biotyping schemes described in this report consist of phenotypic tests that are easy to perform and interpret. They provide good separation of strains within each species and should be very useful for epidemiologic studies of C. jejuni and C. coli infections. They would allow clinical laboratories that are currently unable to perform serotyping to obtain preliminary epidemiological information. In addition, if biotyping and serotyping are combined, this would provide even more specific

epidemiological information about these strains than either technique can alone.

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TABLE 1 Biotyping schemes for C. jejuni and C. coli strains and assignment of strains used in this study.

C. jejuni

Biovar	Alkaline Phosphatase Activity	DNase Activity	Hippurate Hydrolysis	Strains and source[a]
1	+	+	+	B7619: human feces 4164A: human feces 11151: human blood 13136: ovine fetus N82-10A: ovine fetus N82-11A: ovine fetus 61-784: chicken
2	+	-	+	ATCC 33560: bovine feces H840: human feces 12019: human blood 12375: human blood 82214: human blood Holy Cross: human blood 12191: human blood 11641B76: human blood ATCC 33250: human blood ATCC 33251: human blood Veit: ovine fetus
3	+	+	-	H325: human feces
4	-	-	+	PC 340: unknown

Table 1, continued. . . .

C. coli

Biovar	Alkaline Phosphatase Activity	DNase Activity	Growth in minimal medium	Strains and source
1	+	+	+	ATCC 33559: porcine feces C607: porcine feces C602: porcine feces 541: porcine feces H550: human feces 7292B76: human blood 76-3227: human blood C679: chicken feces
2	+	-	+	C699: chicken feces
3	-	-	+	80-15573: porcine feces 81-3592-3: porcine feces
4	-	-	-	80-1184-2: porcine feces

[a]Further information regarding the origin of the strains has been presented by Roop et al. (18).

Table 2. Characteristics useful for differentiating C. jejuni and C. coli strains.[a]

Characteristic	C. jejuni	C. coli
Hippurate hydrolysis	+	-
Growth in minimal medium	-	+
H_2S production, TSI slant	-	+[b]

[a] Roop et al.(18).

[b] TSI slants were freshly prepared and only those that had water of syneresis were used. This water of syneresis in essential in order to demonstrate H_2S production. Slants were incubated at 37°C under an atmosphere of 6% O_2, 5% CO_2 and 89% N_2 for 7 days. C. coli strains produced H_2S at the junction of the butt and slant (18).

Chapter 3

Campylobacter laridis Causing Bacteremia in an Immunocompromised Patient

Abstract

An unusual species, Campylobacter laridis, belonging to the group of nalidixic acid-resistant thermophilic Campylobacter species, was isolated from the blood of a 71-year-old man with multiple myeloma, hyperviscosity syndrome, and renal failure. The organism was first recognized in the laboratory by Gram-stain reaction and resistance to nalidixic acid. The organism differs from C. jejuni and C. coli by its resistance to nalidixic acid, whereas anaerobic growth in the presence of trimethylamine-N-oxide differentiates this organism for other Campylobacter species. Biochemical characterization and DNA homology studies confirmed the identity of this species as being C. laridis. To our knowledge, this is the first recorded case of bacteremia due to C. laridis in humans.

Introduction

The genus Campylobacter consists of several species including C. jejuni, C. coli, C. fetus subspecies fetus, C. fetus subspecies venerealis, C. sputorum subspecies sputorum, C. sputorum subspecies bubulus, C. sputorum subspecies mucosalis, and "C. fecalis" (1). Although all of these species are common veterinary isolates, only C. jejuni, C. coli and C. fetus subspecies fetus are known to cause human disease.

A recently described species, C. laridis, previously described as belonging to the nalidixic acid-resistant thermophilic Campylobacter group (2,3), has been isolated most frequently from gulls of the genus Larus (2), but also from dogs, monkeys, cats, poultry, wildfowl ponds, and rarely, human feces. Although C. jejuni, C. coli, and C. fetus subspecies fetus have been reported to cause bacteremia (4), human isolates of C. laridis have not been associated with disease (3).

We recently isolated C. laridis (HUP1789) from blood cultures of an immunocompromised patient. To our knowledge, this is the first reported case of bacteremia due to this organism.

Case Report

A 71-year-old white man was admitted to the Hospital of the University of Pennsylvania of 9 January for evaluation of fever and abrupt change in mental status. This was the 11th admission for this patient, a retired government clerk who lived in a large city and had no exposure to farm animals. He had a history of peptic ulcer disease and renal cell carcinoma for which he had a left adrenalectomy and nephrectomy in 1979. In 1981 he was diagnosed as having multiple myeloma. At that time serum electrophoresis showed a monoclonal component identified as an IgA kappa at a concentration of 5.3 g/dL. The relative serum viscosity at that time was 3.54 (relative to water; normal, 1.4 to 1.8). The patient was treated with melphalan and prednisone but had only a partial response. The patient initially received peritoneal dialysis but was switched to hemodialysis because of infection.

In March 1982, the monoclonal component returned to 6 g/dL and the patient was treated with doxorubicin and carmustine with no response. This treatment program was complicated by the patient's pancytopenia. From June 1982 to September 1982, he had several episodes of infection including pneumonia that were successfully treated with empiric antimicrobial chemotherapy. The patient also had diffuse bleeding episodes secondary to thrombocytopenia and increased prothrombin time and partial thromboplastin time, one of which required hospitalization. The bleeding was believed to be secondary to inhibition of clotting factors or platelets by the paraprotein. He was treated with transfusions of fresh frozen plasma, cryoprecipitate, and platelets, and was started on prednisone with resolution of bleeding.

Two days before the final admission, the patient was alternately lethargic and agitated, with some degree of disorientation and a temperature of 37.2°C. On the morning of admission, his temperature rose and he became increasingly obtunded. The patient was cachetic and responded only to his name. His blood pressure was 75/50 mm Hg; pulse 108/minute; respiratory rate, 20/minute; temperature, 38.9°C. His neck was stiff but with no focal neurologic deficits. No lymphadenopathy or diarrhea was seen. Initial laboratory findings included a hemoglobin level of 11g/dL and a leukocyte count of 6.1 x $10^3/\mu L$. The differential count showed 49% polymorphonuclear leukocytes, 3% bands, 20% lymphocytes, and 27% plasma cells. Gross flecks of red blood were seen in the stool. The serum calcium level was 10.8 mg/dL (normal, 8.5 to 10.5); total protein, 10 g/dL (normal, 6.1 to 8.5); albumin, 2.0 g/dL (normal, 3.5 to 5.8); creatinine, 10.2 mg/dL; and his liver enzymes were normal. Coagulation studies showed an elevated prothrombin time, partial thromboplastin time, and fibrinogen of 65 mg/dL (normal, 170 to 410). Fibrin split products were not measured. The relative serum viscosity was 5.56 (relative to water; normal, 1.4 to 1.8). Prednisone was continued. He was treated with vancomycin, gentamicin, moxalactam, and penicillin G, and continued on hemodialysis with little improvement. Three days after admission, the patient had a respiratory arrest and died.

Cultures of urine and cerebrospinal fluid taken on admission were negative. Blood cultures grew a Gram-negative, curved rod that did not grow aerobically, grew poorly anaerobically, but grew well in a microaerophilic environment.

Methods

All bacteria were grown on Trypticase soy agar with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Maryland) at 42°C in a microaerophilic atmosphere (CampyPak, BBL Microbiology Systems). The following biochemical tests were done according to published methods (2,3,5): growth at 25°C, 37°C, 42°C; catalase; oxidase, H_2S production (lead acetate paper); sensitivity to nalidixic acid and cephalothin; motility; triple sugar iron agar reaction; tolerance to 1% glycine; growth on 1.5% and 3.5% NaCl agar; tolerance to 1% bile; DNA hydrolysis; nitrate reduction; and anaerobic growth in the presence of trimethylamine-N-oxide hydrochloride. Hippurate hydrolysis was done using the rapid method of Hwang and Ederer (6) described by Luechtefeld and Wang (7). Tolerance to triphenyltetrazolium chloride was done using a microtiter procedure. Bacterial suspensions were adjusted to a 0.5 McFarland standard in brucella broth (BBL Microbiology Systems) and 0.05 mL was inoculated in 0.05 ml of brucella broth with serial twofold concentrations of triphenyltetrazolium chloride (1.5 µg/ml to 1 mg/ml). The plate was incubated at 42°C overnight in a 5% oxygen atmosphere. Wells were watched for growth and production of a red formazan dye that indicated reduction of the triphenyltetrazolium chloride. Alkaline phosphatase activity was determined using a modification of the method described by Hébert and associates (5). Cells were grown in a biphasic system consisting of 50 mL of iron bisulfite pyruvate (FBP) broth (8) and 200 mL of FBP agar (2.5% agar) in a Roux bottle for 48 hours at 37°C and harvested by centrifugation at 13,000 x g for 10 minutes. The cell pellet was washed once with sterile 0.85% saline and resuspended in 0.85%

saline. The cell suspension was adjusted to a turbidity equivalent to a #3 McFarland standard. One half mL of the cell suspension was pipetted into 16 x 125-mm test tubes, 0.5 mL of the alkaline phosphatase reagent added, and the results read after 6 hours incubation at 37°C. Any degree of yellow color was considered positive.

The DNA homology studies were done according to the method of Johnson (9) using the hydroxylapatite method for isolating bacterial DNA and S1 nuclease procedure for homology studies. DNA for homology studies was labeled with ^{125}Iodine according to the method of Selin and associates (10). Homology studies were done with the following strains: C. laridis C729 (human feces); C. jejuni ATCC 33560 (cattle feces), Holy Cross (human blood), H325 (human feces), 82214 (human blood), and H840 (human feces); C. coli ATCC 33559 (pig feces), 76-3227 (human blood), H550 (human feces), 7292B76 (human blood), and 541 (pig feces); C. fetus subspecies fetus ATCC 27374 (fetal sheep brain), 1510MB (human blood), Pacemaker (human blood), and Yale (human blood); C. fetus subspecies venerealis UMHS (bovine abortion); "C. hyointestinalis" 80-4577-4 (pig feces) and 81-13087 (pig feces); "C. fecalis" 14479 (sheep feces) and 11411 (sheep feces); and aerotolerant Campylobacter strains 02790 (pig fetus) (11) and 02824 (cattle fetus).

Two blood culture sets were drawn when the patient was admitted. Gram stain of the blood culture bottles showed the presence of a curved, Gram-negative rod, suggestive of Campylobacter species. Subculture to blood agar showed no growth under aerobic conditions or with an increased $C0_2$ atmosphere. Growth using anaerobic conditions was poor. The organism was isolated using a microaerophilic environment.

Results

Table 1 shows the results of biochemical characterization of this blood isolate. The key results that differentiated C. laridis from the other Campylobacter species were anaerobic growth in the presence of trimethylamine-N-oxide and resistance to nalidixic acid. Other tests that differentiated it from other species included hippurate hydrolysis (C. laridis, negative; C. jejuni, positive), growth on 1.5% NaCl medium, and sensitivity to triphenyltetrazolium chloride.

Table 2 shows the results of DNA homology studies. The study strain showed 91% DNA homology with a referenc strain of C. laridis, whereas less that 10% DNA homology with reference strains was shown to other Campylobacter species.

Antimicrobial susceptibility testing was done by disk diffusion with the following results: penicillin, 10 U, no zone; tetracycline, 30 µg, 14-mm zone; erythromycin, 15 µg, 29-mm zone; ampicillin, 10 µg, no zone; oleandomycin, 15 µg, 30-mm zone; chloramphenicol, 30 µg, 24-mm zone; clindamycin, 10 µg, 30-mm zone; cephaloridine, 5 µg, 18-mm zone; cefoxitin, 30 µg, 15-mm zone; and cephalozin, 30 µg, no zone.

Discussion

We report the first case of C. laridis causing bacteremia in humans. Although isolation from the blood of our patient does not implicate this bacterium as the cause of death, it appears to be a clinically significant isolate because of the patients's fever, clinical suspicion of sepsis, and isolation of the organism from two blood culture bottles at the time of hospitalization. The source of this organism is not known. C. laridis has been isolated only rarely from human feces, without known clinical significance. Bleeding in the gastrointestinal tract, along with the patient's other bleeding problems, suggests that the portal of entry may have been in the gastrointestinal tract. Why this particular organism rather than some other commoner enteric flora entered the blood is not known. Unfortunately, stool cultures were not done on this patient to confirm this hypothesis.

Skirrow and Benjamin (2) described a group of 42 thermophilic Campylobacter strains belonging to the group of nalidixic acid-resistant thermophilic Campylobacter species isolated from different sources. The commonest source was from gulls of the genus Larus (L. argentatus, L. fuscus, L. ridibunous). Other isolates included two human strains isolated from feces, and strains from dogs, monkeys, cats, poultry, and water. Benjamin and associates (3) recently characterized ten strains using biochemical and DNA homology studies; members of this group appeared to belong to a separate species within the genus Campylobacter and the name Campylobacter laridis was proposed for these isolates. Our DNA studies also confirm the DNA homology studies of Benjamin and associates (3).

Campylobacter laridis can readily be distinguished from other thermophilic species based on resistance to nalidixic acid. As Benjamin and associates (3) point out, other tests may be useful and more reliable for its identification because occasional strains of C. jejuni may be resistant to nalidixic acid. Of the tests used in their scheme, anaerobic growth in the presence of trimethylamine-N-oxide is the most definitive test for the identificaton of C. laridis. Other Campylobacter species have not been shown to grow in its presence. According to Benjamin and associates (3), trimethylamine-N-oxide is a product of urinary excretion in marine fish and may facilitate growth of the organism in the gut of animals, such as gulls, that eat them. This case is an example of the increasing clinical importance of unusual, primarily nonhuman, pathogens in the immunosuppressed patient.

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Table 1. Comparison of characteristics of human *Campylobacter laridis* isolate to other *Campylobacter* species[a].

Characteristic	*C. laridis* (HUP 1789)	*C. laridis*[b]	*C. jejuni*[b]	*C. coli*[b]
Growth at:				
25°C	-	-	-	-
37°C	+	+	-	-
42°C	+	+	+	+
Catalase	+	+	+	+
Oxidase	+	+	+	+
Hippurate hydrolysis	-	-	+	-
H_2S production:				
Lead acetate strip	+	+	+	+[c]
Triple sugar iron agar	-	-	-	+
DNA hydrolysis	-	-	d	d
Alkaline phosphatase	-	-	+	d
Nitrate reduction	+	+	+	+
Motility	+	+	+	+
Growth in:				
1% glycine	+	+	+	+
1.5% NaCl (plates)	+	+	-	-
3.5% NaCl	-	-	-	-
1% bile	+	+	+	+
0.1% TMAO (anaerobically)	+	+	-	-
Sensitivity to:				
nalidixic acid (30 µg disk)	-	-	+	+
cephalothin (10 µg disk)	-	-	-	-
triphenyltetrazolium chloride	+	+	-	-

Table 1, continued.....

[a]+ = 90% to 100% of strains positive; d = 26% to 75% of strains positive; - = 0% to 10% of strains positive.

[b]Reference strains.

[c]If there is sufficient water of syneresis present, strains of C. coli on triple sugar iron agar slants incubated at 6% O_2, 5% CO_2 will produce a positive H_2S reaction at the junction of the slant and butt in 2 to 4 days. The reaction resembles the characteristic "mustache" produced by Salmonella typhi.

Table 2. DNA homology of Campylobacter laridis (HUP1789) strain with reference strains of other Campylobacter species.

Organism	Percent DNA homology with strain HUP1789
C. laridis C729	91
C. jejuni ATCC 33560 Holy Cross H325 82214 H840	10[a]
C. coli ATCC 33559 76-3227 H550 7292B76 541	9
C. fetus subspecies fetus ATCC 27374 1510MB Pacemaker Yale	3
C. fetus subspecies venerealis UMHS	3
"C. hyointestinalis" 80-4577-4 80-13087	3
"C. fecalis" 14479 11411	10
Aerotolerant Campylobacter[b] 02790 02824	3

[a]Average relatedness of strains tested.

[b]See reference 11.

Chapter 4

DNA Homology Studies of the Catalase-Negative Campylobacters and *Campylobacter "fecalis"* and an Emended Description of *Campylobacter sputorum*

Abstract

Twenty four strains of catalase-negative campylobacters and five strains of Campylobacter "fecalis" were examined by DNA homology. These organisms formed four distinct DNA homology groups corresponding to C. sputorum, C. mucosalis, C. concisus and a currently unnamed group referred to as the "catalase-negative or weak" (CNW) strains. These strains were further characterized to determine which phenotypic characteristics provide the most reliable identification at the species and biovar levels. H_2S production in SIM medium and on TSI slants, the ability to grow in the presence of 1% oxgall, 1% glycine or 3.5% NaCl, growth at 25°C, alkaline phosphatase activity, sensitivity to cephalothin, a requirement for H_2 or formate for microaerophilic growth and a requirement for either H_2 and fumarate or formate and fumarate for anaerobic growth proved to be the most useful characteristics for properly identifying these strains.

Introduction

For many years, the genus Campylobacter has been divided into two broad groups based on the the presence or absence of catalase activity (2). The organisms of human and veterinary medical importance fall into the catalase-positive group and the catalase-negative organisms are for the most part considered to be commensals. Some catalase-negative campylobacters have been isolated from disease states in man and animals, but at the present their role in these conditions is not well defined (6,14,18,24,28).

Catalase-negative campylobacters are currently placed into one of three groups - Campylobacter sputorum, C. concisus (28), and an unnamed group referred to as the catalase-negative or weak (CNW) group that has been recently isolated from the feces of dogs and cats (6,24). C. sputorum is further divided into three subspecies. C. sputorum subspecies sputorum is a commensal found in the human oral cavity (16,27) and occasionally isolated from normal human feces (12). C. sputorum subspecies bubulus is found in the reproductive tracts of cattle and sheep and is not considered to be pathogenic (27). C. sputorum ssp. mucosalis, on the other hand, has been implicated in porcine intestinal adenomatosis (PIA) (14); however, a recent report (18) raises questions about the ability of this organism alone to produce PIA. We recently submitted a report (Roop, R. M. II, R. M. Smibert, J. L. Johnson and N. R. Krieg, "Campylobacter mucosalis (Lawson, Leaver, Pettigrew and Rowland, 1981) sp. nov, comb. nov.: emended description", Int. J. Syst. Bacteriol., in press) in which we presented DNA homology data showing that C. sputorum subsp. mucosalis strains are not related to either C.

sputorum subsp. sputorum or C. sputorum subsp. bubulus at the species level. We have proposed the name C. mucosalis for these strains and hereafter we will refer to them as such. C. concisus has been isolated from the gingival crevices of humans suffering from peridontal disease, but again the role of this organism in this condition is unknown (28).

Campylobacter "fecalis", a catalase-positive organism isolated most often from the feces of sheep (4), has many of the phenotypic characteristics that are associated with catalase-negative campylobacters, i.e. nitrite reduction, H_2S production in SIM medium (23) and there has been speculation that this organism may be more closely related to the catalase-negative campylobacters than it is to the catalase-positive group (8).

In this report, the relationships between the various catalase-negative campylobacters, as well as the relationship of C. "fecalis" to these organisms, are assessed at the species level by means of DNA homology. Phenotypic characteristics useful for differentiating these organisms are also presented.

Materials and methods

Organisms. The sources of the catalase-negative and C. "fecalis" strains used in this study are listed in Table 1. The sources of the catalase-positive reference strains, with the exception of C. nitrofigilis, have been published previously (23). C. nitrofigilis ATCC 33309 was obtained from the American Type Culture Collection. Type strains, if they were available, were used as sources of reference DNA in the homology studies. In the case of C. sputorum subsp. sputorum, the type strain ER-33 (16) is no longer extant. C. sputorum subsp. sputorum strain VPI S-17 has all of the phenotypic characteristics described for C. sputorum subsp. sputorum (16), and has been used as a reference strain for C. sputorum subsp. sputorum by us previously (Roop et al., "Campylobacter mucosalis (Lawson, Leaver, Pettigrew and Rowland, 1981) sp. nov., comb. nov.: emended description", Int. J. Syst. Bacteriol., in press), as well as by others (9,28).

We obtained the type strain of C. concisus, ATCC 33237, from the American Type Culture Collection, but when it was checked for purity by streaking, four different colony types were found, of which only two corresponded to the description of C. concisus. These two colony types are designated ATCC 33237B and ATCC 33237C in this report. One of the other colonies yielded an organism similar in morphology to C. concisus, but which was catalase-positive. This organism was designated ATCC 33237A. The remaining colony type was rough and black and resembled an actinomycete colony, no effort to further characterize this organism was made. C. concisus strain VPI 13086 (= F/S 484), which was used as the reference strain in this

study, was designated by Tanner et al. (28) as the type strain for C. concisus. We obtained this strain, which yielded only a single colony type, from the culture collection of the VPI Department of Anaerobic Microbiology.

Type strains have not been formally proposed for C. "hyointestinalis" (5) or C. "fecalis" (4), but the reference strains we used were those suggested by the authors who first reported these organisms. In the case of the CNW strains, we used CG-1 as a reference strain because it yielded a greater amount of DNA than did strain C231.

Growth conditions. Stock cultures of C. sputorum subsp. sputorum, C. sputorum subsp. bubulus and the CNW strains, as well as the catalase-positive campylobacter strains used in this study, were maintained in semisolid Brucella medium as previously described (23). C. nitrofigilis (20) was maintained in semisolid Brucella medium supplemented with 1% NaCl at 30°C with weekly transfer. C. mucosalis and C. concisus stock cultures were maintained in semisolid Brucella supplemented with 0.3% fumaric acid. The pH of this medium was adjusted to 7.0 with solid KOH before sterilizing, and these cultures were incubated at 37°C under an atmosphere of 6% O_2, 5% CO_2, 15% H_2 and 74% N_2 with weekly transfer.

The methods used for growing cultures for DNA isolation have been described previously (23; Roop et al., "Campylobacter mucosalis (Lawson, Leaver, Pettigrew and Rowland, 1981) sp. nov., comb. nov.: emended description", Int. J. Syst. Bacteriol., in press).

DNA isolation, DNA homology experiments and determination of DNA base composition. DNA was extracted and purified by the hydroxylapatite (HA) procedure described by Johnson (11). The S-1 nuclease procedure described by Johnson (11) was used for the DNA homology experiments. Homology experiments were performed under "optimal" conditions, i.e., 25°C below the thermal melting point (T_m) of the DNA (18). DNA from reference strains was labeled in vitro with ^{125}I according to the method of Selin et al. (25).

The thermal melting point (T_m) of the DNA of representative strains from each homlogy group was determined using the method described by Johnson (11) and the mole percent guanine plus cytosine (mol% G + C) was determined using the equation of Mandel et al. (17). DNA from Escherichia coli strain b, which has a mol% G + C of 51, was used as a reference.

Physiological characteristics. Catalase activity, oxidase activity, hippurate hydrolysis, nitrate and nitrite reduction, growth in the presence of 1% glycine, 1% oxgall, 3.5% NaCl, growth in minimal medium, anaerobic growth in 0.1% trimethylamine-N-oxide (TMAO), aerobic growth, H_2S production in Sulfide-Indole-Motility (SIM) medium, H_2S production on Triple Sugar Iron (TSI) agar slants, growth on agar plates containing 1.5% NaCl, DNase activity and sensitivity to nalidixic acid and cephalothin were determined using procedures that have been described in a previous report (23) with the exception that all agar plates were supplemented with 3 g/L fumaric acid and the pH of the supplemented agar media adjusted to 7.0 with solid KOH. All plates and TSI agar slants were incubated under an atmosphere of 5% O_2, 5% CO_2, 15% H_2 and 75% N_2. Cultures grown in semisolid media which were used

for testing the physiological characteristics (except anaerobic growth in 0.1% TMAO) of C. mucosalis and C. concisus strains were incubated under an atmsophere of 6% O_2, 5% CO_2, 15% H_2 and 74% N_2. Cultures grown in semisolid media used for testing the physiological characteristics of the other campylobacters in this study were incubated aerobically. Alkaline phosphatase activity was determined using a previously described procedure (23), but the basal medium was Brucella medium supplemented with 0.3% fumarate (BF medium) for the C. sputorum subsp. sputorum, C. sputorum subsp. bubulus and "C. fecalis" strains. BF medium supplemented with 0.2% formate was used for the C. mucosalis and C. concisus strains. Microaerophilic growth was tested on Brucella agar plates under atmospheres of 5% O_2, 5% CO_2 and 90% N_2 and 5% O_2, 5% CO_2, 15% H_2 and 75% H_2. Anaerobic growth was tested on Brucella agar plates supplemented with 0.3% ammonium formate and on unsupplemented Brucella agar plates incubated in anaerobe jars under atmospheres of 10% H_2, 5% CO_2 and 85% N_2 with a Pd catalyst or 5% CO_2 and 95% N_2 without a catalyst. In the latter case the jars were exhaustively flushed with the CO_2/N_2 mixture to ensure anaerobic conditions. Anaerobic growth was also determined in pre-reduced anaerobically-sterilized Peptone Yeast Extract Glucose broth supplemented with hemin and vitamin K (PYG-HK) (10) to which had been added either 0.2% fumarate or 0.2% fumarate and 0.2% formate.

Results

The C. sputorum subsp. sputorum, C. sputorum subsp. bubulus and C. "fecalis" strains used in this study all showed a high level of DNA homology (72-100%) with the type and reference strains for these species and subspecies, viz., C. sputorum subsp. sputorum S-17, C. sputorum subsp. bubulus ATCC 33562 and C. "fecalis" 11363, respectively. However, they did not show any significant DNA homology with any of the other reference strains used, including C. mucosalis NCTC 11,000. The C. mucosalis, C. concisus and CNW strains used each formed their own distinct DNA homology groups and did not show any significant DNA homology with any of the other reference strains used (Table 2). None of the catalase-negative strains used showed any significant DNA homology with the catalase-positive reference strains used, with the exception of C. "fecalis" (Table 3).

The mol% G + C values determined for representative strains from each of the four DNA homology groups ranged from 31 for C. sputorum subsp. bubulus ATCC 33562 to 39 for C. mucosalis NCTC 11,000 and C. concisus ATCC 33237B and C (Table 4).

The biochemical and physiological characteristics for all of the strains are listed in Table 5.

Discussion

The results of our DNA homology studies show that C. sputorum subsp. sputorum, C. sputorum subsp. bubulus and C. "fecalis" strains are related at the species level. C. sputorum has historically been divided into two subspecies. C. sputorum subsp. sputorum contains the strains that are isolated as commensals from the human mouth (16,27) and occasionally, from normal human feces (12). C. sputorum subsp. bubulus is part of the normal genital flora of cattle and sheep (27). These two subspecies can be easily differentiated in the laboratory by their abilities to grow in the presence of 1% oxgall (C. sputorum subsp. sputorum) or 3.5% NaCl (C. sputorum subsp. bubulus) (27, this study). C. "fecalis" has been isolated from sheep feces and from the genitalia of cattle, but has never been associated with disease in man or animals (26,27). Al-Mashat and Taylor (1) reported the production of enteritis in calves that had been inoculated orally with C. "fecalis", but the description of the organism more closely resembled that of C. "hyointestinalis" than "C. fecalis" and the organism may have been misidentified. C. "hyointestinalis" has been isolated from calves with enteritis (22, 29).

Because C. sputorum subsp. sputorum, C. sputorum subsp. bubulus and C. "fecalis" strains all share very high levels of DNA homology (>72%), we propose that all three of these organisms belong in a single species, C. sputorum. However, because there appears to be some host specificity among these organisms, we believe there are practical reasons for placing these organisms into three biovars, namely C. sputorum biovar sputorum, C. sputorum biovar bubulus and C. sputorum biovar fecalis. In this study, 3

strains of C. sputorum isolated from humans were used and all three were identified biochemically as strains of C. sputorum biovar sputorum. Two of these strains were from clinical samples, one from a leg abscess and the other from a diarrheic stool sample from an infant. Whether C. sputorum biovar sputorum strains will be shown in the future to be important as human pathogens remains to be seen; at present they are not routinely looked for in clinical samples. In the event that they have some clinical significance, then a means of differentiating the human strains from the animal strains would become important. In addition, because the type strain of C. sputorum biovar sputorum is no longer extant, we propose VPI S-17 as the neotype strain for C. sputorum. This strain has all of the phenotypic characteristics described for the type strain of C. sputorum biovar sputorum (16), has been deposited with the American Type Culture Collection and has been used by us (Roop et al., "Campylobacter mucosalis (Lawson, Leaver, Pettigrew and Rowland, 1981) sp. nov., comb. nov.: emended description", Int. J. Syst. Bacteriol., in press) and others (9,28) as a reference strain for C. sputorum biovar sputorum.

All of the C. sputorum strains except C. sputorum biovar sputorum Harkness produced H_2S on TSI agar slants and in SIM medium. These strains were also able to grow at 42°C, but not at 25°C, all were sensitive to cephalothin and all but C. sputorum biovar sputorum S-17 were resistant to nalidixic acid. All of the strains tested reduced nitrate to nitrite, however, only 9 of 21 strains reduced nitrite.

We reported earlier that C. sputorum biovar fecalis strains would not grow on Brucella agar plates containing 1.5% NaCl under an atmosphere of 6% O_2, 5% CO_2 and 89% N_2 (23). During the course of the study reported here,

these strains were retested on Brucella agar to which fumarate had been added and under different atmospheric conditions (6% O_2, 5% CO_2, 15% H_2 and 74% N_2). Under these conditions, C. sputorum biovar fecalis strains were able to grow in the presence of 1.5% NaCl.

C. sputorum biovar bubulus and C. sputorum biovar fecalis strains could be distinguished from C. sputorum biovar sputorum by their ability to grow in 3.5% NaCl and inability to grow in 1% oxgall. Except for the presence of catalase activity, C. sputorum biovar fecalis strains are identical in their phenotypic characteristics to C. sputorum biovar bubulus strains (23, this study). These characteristics also make C. sputorum biovar bubulus and C. sputorum biovar fecalis strains different from any of the other campylobacters used in this study.

Based on the results given above, we propose that the description of Campylobacter sputorum given in Bergey's Manual of Systematic Bacteriology (27) be emended to state "catalase activity varies among strains".

The C. mucosalis strains all showed a high level of DNA homology with the type strain of C. mucosalis NCTC 11,000 but no significant homology with any of the other campylobacters tested. This indicates that these strains represent a distinct species and are not a subspecies of C. sputorum.

All of the C. mucosalis strains grew at both 25 and 42°C and required either H_2 or formate for microaerophilic growth and H_2 and fumarate or formate and fumarate for anaerobic growth. Lawson et al. (13,15) have also reported that these organisms produced a distinctive yellow pigment, which is best seen when a colony is smeared on a piece of white paper (15). In addition, Moss et al. (21) recently reported that two strains of C. mucosalis

had moderate amounts of lauric acid in their cellular fatty acid profiles, whereas this fatty acid was not present in the other Campylobacter species that they tested.

Nitrite reduction has been reported as a useful differential test for both C. mucosalis and C. sputorum strains (13,15,16). However, only 3 of 8 C. mucosalis strains we tested reduced nitrite, and as mentioned earlier, only 9 of 21 of our C. sputorum strains reduced nitrite. This discrepancy may be due to the fact that in this study, Brucella medium was used as the basal medium, but in the previously reported studies either thioglycollate medium supplemented with 0.2% yeast extract (16) or nitrate broth supplemented with 5% inactivated horse serum (13, 15) was used as the basal medium. From these results, it appears that the choice of basal medium may have an effect on the results of the nitrite reduction test.

C. mucosalis was first isolated from the lesions of porcine intestinal adenomatosis (PIA) (14) and at that time it was believed to be the causative agent of this disease. However, recent attempts to reproduce the disease by inoculating pigs with C. mucosalis alone have been unsuccessful (18). It has been suggested that more than one Campylobacter species may be involved in this disease, and that C. "hyointestinalis" may also play a role (18).

C. concisus strains all demonstrated a high degree of DNA homology with the reference strain, VPI 13086, but were not shown to be highly related to any of the other campylobacters tested. This is in agreement with Tanner et al. (28), who used DNA homology experiments to show that these organisms were not related to C. sputorum subsp. sputorum or C. sputorum subsp. bubulus at the species level.

All of the C. concisus and C. mucosalis strains tested required either H_2 or formate for microaerophilic growth and either H_2 and fumarate or formate and fumarate for anaerobic growth. The C. concisus strains did not grow at 25°C and were resistant to cephalothin, whereas the C. mucosalis strains did grow at 25°C and were sensitive to cephalothin.

Although C. concisus strains ATCC 33237B and ATCC 33237C were isolated from the same culture, ATCC 33237C is either a variant of ATCC 33237B, or two different strains of C. concisus were present in the culture we received from the American Type Culture Collection. ATCC 33237B showed 100% DNA homology with C. concisus 13086, and both of these strains grew on agar plates containing 1.5% NaCl, while ATCC 33237C showed 89% homology with C. concisus 13086 and would not grow on agar plates containing 1.5% NaCl. Strain ATCC 33237A, which was catalase-positive, did not not show any significant DNA homology with any of the reference strains used in this study (data not shown). C. concisus was originally isolated from patients suffering from periodontal disease (28). The role of this organism in this condition is at present unknown.

Sandstedt et al. (24) in Sweden isolated catalase-negative or weakly catalase-positive campylobacters from the feces of dogs and termed them CNW strains. These strains most closely resembled C. coli in their phenotypic characteristics besides catalase activity, but were shown to be unrelated to C. fetus, C. jejuni, C. coli or C. sputorum subsp. bubulus at the species level by DNA homology (24). Similar organisms were later isolated in the U. S. (3,6) from dogs and cats, both healthy and suffering from diarrhea. We used one CNW strain, C231, and one of the U. S. strains, CG-1, in this study. Strain

231 showed 87% DNA homology with strain CG-1, which indicates that these strains are related at the species level.

The CNW strains used in the present study were phenotypically different from the other catalase-negative campylobacters in several respects. The CNW strains did not produce H_2S on SIM medium or TSI slants, did not grow in the presence of 1% glycine and demonstrated alkaline phosphatase activity. These are the only campylobacters we have tested, except some C. coli and C. jejuni strains (23), that are positive for alkaline phosphatase activity. The two strains tested were very strongly positive, turning the reaction mixture bright yellow in 2 hours.

Although the CNW strains have been isolated from dogs and cats suffering from diarrhea (3,6), the importance of these strains in these conditions is not currently known.

In summary, we have investigated the genetic relatedness of the catalase-negative campylobacters and C. "fecalis" with each other, as well as their relatedness to other Campylobacter species, by means of DNA homology experiments. In addition, we characterized these strains phenotypically and the tests that we found to be the most reliable for identification at the species and biovar levels are in Table 6.

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TABLE 1. Catalase-negative Campylobacter and C. "fecalis" strains used in this study[a]

Species and strain	Source	Origin
C. sputorum subsp. sputorum		
S-17[NT]	R. M Smibert, Department of Anaerobic Microbiology, Virginia Polytechnic Institute, Blacksburg, Va.	Human: oral
Harkness	J. Harkness, St. Vincent's Hospital Darlinghurst, Australia	Human: leg abscess
VA-1	L. Albano, Veterans Administration Hospital, West Haven, Conn.	Human: feces
C. sputorum subsp. bubulus		
ATCC 33562[T]	American Type Culture Collection	Bovine
861	R. M. Smibert	Bovine: vaginal mucus
867	R. M. Smibert	Bovine: vaginal mucus
53103	Collection of the Institute Pasteur, Paris, France	Bovine
11763	B. D. Firehammer, Veterinary Research Laboratory, Montana State University, Bozeman, Montana	Bovine: vaginal mucus
11764	B. D. Firehammer	Bovine: bull prepus
11766	B. D. Firehammer	Bovine: vaginal mucus
11767	B. D. Firehammer	Bovine: vaginal mucus

Table 1, continued.....

Species and strain	Source	Origin
C. "fecalis"		
ATCC 33709	B. D. Firehammer	Ovine: feces
11362	B. D. Firehammer	Ovine: feces
11411	B. D. Firehammer	Ovine: feces
14223	B. D. Firehammer	Ovine: feces
14479	B. D. Firehammer	Ovine: feces
"C. mucosalis"		
NCTC 11,000[T]	G. H. K. Lawson, Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Midlothian, Scotland	Porcine: intestinal adenomatosis (IA) lesion
79-12009	G. E. Ward, Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, Minnesota	Porcine: IA lesion
P-9681C	J. Hayes, Purdue University School of Veterinary Medicine, West Lafayette, Indiana	Porcine: IA lesion
P-10546C	J. Hayes	Porcine: IA lesion
P-5007	J. Hayes	Porcine: IA lesion
P-11508	J. Hayes	Porcine: IA lesion
P-5494	J. Hayes	Porcine: IA lesion
P-10742	J. Hayes	Porcine: IA lesion

Table 1, continued.....

Species and strain	Source	Origin
C. concisus		
13086	A. C. R. Tanner, Forsyth Dental Center, Boston, Mass.	Human: periodontal disease
E3X-22	R. M. Smibert	Human: experimental gingivitis
ATCC 33237B	American Type Culture Collection	Human: periodontal disease
ATCC 33237C	American Type Culture Collection	Human: periodontal disease
CNW strains		
CG-1	C. Gebhart, Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, Minnesota	Canine: feces
C231	K. Sandstedt, Department of Bacteriology, National Veterinary Institute, Uppsala, Sweden	Canine: feces

[a]The superscripts ^{NT} and ^{T} indicate neotype strain (see Discussion) and type strain, respectively.

TABLE 2. DNA homology of catalase-negative campylobacters and "C. fecalis"

Unlabeled DNA from strain	% Homology to DNA of reference strains					
	C. sputorum subsp. sputorum S-17	C. sputorum subsp. bubulus ATCC 33562	C. "fecalis" ATCC 33709	C. mucosalis NCTC 11,000	C. concisus 13086	CNW strains CG-1
GROUP I						
C. sputorum subsp. sputorum						
S-17	100	80	90	3	4	2
VA-1	99	85	89	3	4	4
Harkness	75	79	90	3	3	2
C. sputorum subsp. bubulus						
ATCC 33562	89	100	91	5	3	3
53103	89	100	88	4	6	2
867	93	84	92	3	3	3
861	92	82	90	4	4	2
11763	100	80	86	3	3	2
11764	89	80	86	2	4	2
11766	93	78	100	4	5	2
11767	84	72	84	2	4	2
C. "fecalis"						
ATCC 33709	100	80	100	3	3	3
11411	99	87	100	4	5	3
11362	96	79	97	3	3	3
14223	100	80	96	4	4	2
14479	90	80	85	4	3	2

Table 2, continued.....

Unlabeled DNA from strain	% Homology to DNA of reference strains					
	C. sputorum subsp. sputorum S-17	C. sputorum subsp. bubulus ATCC 33562	C. "fecalis" ATCC 33709	C. mucosalis NCTC 11,000	C. concisus 13086	CNW strains CG-1
GROUP II						
C. mucosalis						
NCTC 11,000	12	5	3	100	9	3
P5007	9	5	4	96	9	3
P5494	8	5	3	96	10	3
P11508	9	4	3	96	11	3
P10546C	7	4	3	95	9	2
79-12009	14	4	4	92	9	3
P10742	10	4	3	92	11	3
P9681C	12	4	3	92	10	3
GROUP III						
C. concisus						
13086	10	4	3	9	100	2
ATCC 33237B	8	4	3	8	100	3
ATCC 33237C	5	2	3	6	89	2
E3X-22	8	3	3	9	78	2
GROUP IV						
CNW strains						
CG-1	11	7	6	2	3	100
C231	5	3	3	3	2	87

TABLE 3. DNA homology of catalase-positive campylobacter reference strains with reference strains of catalase-negative campylobacters and C. "fecalis".

Unlabeled DNA from strain	% Homology to DNA of reference strains					
	C. sputorum subsp. sputorum S-17	C. sputorum subsp. bubulus ATCC 33562	C. "fecalis" ATCC 33709	C. mucosalis NCTC 11,000	C. concisus 13086	CNW strains CG-1
C. fetus subsp. fetus						
ATCC 27374	5	4	4	4	4	3
1510MB	13	5	3	3	3	3
C. fetus subsp. venerealis						
ATCC 19438	7	6	3	4	5	2
B24MK	18	6	4	3	4	4
C. jejuni						
ATCC 33560	4	5	3	6	3	13
H840	14	5	1	2	2	15
C. coli						
ATCC 33559	5	4	8	2	2	10
541	7	3	1	2	1	9
C. laridis						
NCTC 11352	8	4	4	2	3	7
C. nitrofigilis						
ATCC 33309	9	3	1	2	1	8

Table 3, continued.....

Unlabeled DNA from strain	% Homology to DNA of reference strains					
	C. sputorum subsp. *sputorum* S-17	*C. sputorum* subsp. *bubulus* ATCC 33562	*C.* "*fecalis*" ATCC 33709	*C. mucosalis* NCTC 11,000	*C. concisus* 13086	CNW strains CG-1
"*C. hyointestinalis*" 80-4577-4	18	10	9	5	5	2
Aerotolerant campylobacters 02790	9	3	0	2	2	3

TABLE 4. Mol% G + C values for representative catalase-negative campylobacter and C. "fecalis" strains.

Species	Strain	T_m(°C)[a]	Mol% G + C[b] (±1%)
C. sputorum			
subsp. sputorum	S-17	80.9	32
subsp. bubulus	ATCC 33562	80.6	31
C. "fecalis"	ATCC 33709	81.2	33
C. mucosalis	NCTC 11,000	84.3	39
	79-12009	83.9	38
C. concisus	ATCC 33237B	84.3	39
	ATCC 33237C	84.3	39
	E3X-22	84.1	38
CNW strains	CG-1	82.4	35
	C231	82.7	36

[a]Values normalized for 1 X SSC (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0).

[b]Calculated using the equation of Mandel et al. (17).

TABLE 5. Biochemical and physiological characteristics of the Campylobacter strains used in this study.

Characteristic	C. sputorum biovar sputorum	C. sputorum biovar bubulus	C. sputorum biovar fecalis	C. mucosalis	C. concisus	CNW strains
DNA homology group	I	I	I	II	III	IV
No. of strains tested	3	8	5	8	3	2
Oxidase	3[a]	8	5	8	4	2
Catalase	0	0	5	0	0	0
Nitrate reduction	3	8	5	8	3	2
Nitrite reduction	1	3	5	3	3	0
H_2S production,						
SIM medium	2	8	5	8	3	0
TSI slants	2	8	5	8	3	0
Growth in:						
1% oxgall	3	0	0	8	3	2
1% glycine	3	8	5	8	3	0
1.5% NaCl (plates)	2	8	5	0	2	0
3.5% NaCl	0	7	5	0	0	0
minimal medium	0	1	1	0	0	0
Anaerobic growth in 0.1% TMAO	1	8	5	0	0	0
Aerobic growth	0	0	0	0	0	0

TABLE 5, continued.....

Characteristic	*C. sputorum* biovar sputorum	*C. sputorum* biovar bubulus	*C. sputorum* biovar fecalis	*C. mucosalis*	*C. concisus*	CNW strains
Microaerophilic growth	3	8	5	0	0	2
Microaerophilic growth with H_2	2	8	5	8	3	2
Anaerobic growth with fumarate	3	8	5	0	0	2
Anaerobic growth with formate and fumarate	3	8	5	8	3	2
Growth at:						
25°C	0	0	0	8	0	2
42°C	3	8	5	8	3	2
Hippurate hydrolysis	0	0	0	0	0	0
Alkaline phosphatase activity	0	0	0	0	0	2
DNase activity	1	3	5	1	0	1
Sensitive to:						
nalidixic acid[b]	2	1	0	1	0	2
cephalothin[c]	3	8	5	8	0	2

TABLE 5, continued.....

[a]Values given are the number of strains giving a positive reaction or showing sensitivity to an antimicrobial agent.

[b]30 µg disks.

[c]30 µg disks.

TABLE 6.[a] Differential characteristics for *C. sputorum*, *C. mucosalis*, *C. concisus* and the CNW strains.

Characteristic	*C. sputorum* biovar sputorum	*C. sputorum* biovar bubulus	*C. sputorum* biovar fecalis	*C. mucosalis*	*C. concisus*	CNW strains
Catalase activity	-	-	+	-	-	-[b]
H_2S production,						
SIM medium	d	+	+	+	+	-
TSI slant	d	+	+	+	+	-
Growth in:						
1% oxgall	+	-	+	+	+	+
1% glycine	+	+	+	+	+	-
3.5% NaCl	-	[+]	+	-	-	-
Growth at:						
25°C	-	-	-	+	-	-
H_2 or formate required for microaerophilic growth	-	-	-	+	+	-
H_2 and fumarate or formate and and fumarate required for anaerobic growth	-	-	-	+	+	-

TABLE 6, continued.....

Characteristic	*C. sputorum* biovar sputorum	*C. sputorum* biovar bubulus	*C. sputorum* biovar fecalis	*C. mucosalis*	*C. concisus*	CNW strains
Alkaline phophatase activity	-	-	-	-	-	+
Sensitive to cephalothin	+	+	+	+	-	+

[a]Symbols: + = 90-100% of the strains positive, [+] = 76-89% of strains positive, d = 25-75% of the strains positive, - = 0-10% of the strains positive.

[b]Some strains may show a delayed, weak reaction (24).

Chapter 5

Campylobacter mucosalis (Lawson, Leaver, Pettigrew and Rowland, 1981) sp. nov., comb. nov.: Emended Description

Abstract

Seven strains of Campylobacter sputorum subsp. mucosalis and reference strains of C. fetus, C. jejuni, C. coli, C. laridis, C. "hyointestinalis", C. "fecalis", C. sputorum subsp. sputorum, C. sputorum subsp. bubulus, C. nitrofigilis, aerotolerant campylobacters and CNW strains were compared to the type strain of C. sputorum subsp. mucosalis NCTC 11,000^T in DNA hybridization experiments. NCTC 11,000^T showed a high level of DNA homology with all of the C. sputorum subsp. mucosalis strains tested, but no significant homology with any of the other reference strains used, including those of C. sputorum subsp. sputorum and C. sputorum subsp. bubulus. Based on these observations we propose that C. sputorum subsp. mucosalis be reclassified as C. mucosalis sp. nov., comb. nov.

Introduction

In 1974, Lawson and Rowland isolated microaerophilic, Gram-negative, curved bacteria from the lesions of porcine intestinal adenomatosis (PIA). These organisms resembled C. sputorum in their morphologic and phenotypic characteristics and were given the name Campylobacter sputorum subsp. mucosalis (7,8). C. sputorum subsp. mucosalis differs from C. sputorum subsp. sputorum and C. sputorum subsp. bubulus in two respects. First, C. sputorum subsp. mucosalis requires either H_2 or formate as an electron donor for microaerophilic or anaerobic growth (8) whereas C. sputorum subsp. sputorum and C. sputorum subsp. bubulus grow under either microaerophilic or anaerobic conditions without H_2 or formate. However, growth of C. sputorum subsp. sputorum and C. sputorum subsp. bubulus may be enhanced by these compounds (21). Second, the DNA base composition reported for C. sputorum subsp. mucosalis is 34 mol% G + C (8), compared with 30-32 mol% G + C reported for C. sputorum subsp. sputorum and C. sputorum subsp. bubulus (21).

The purpose of this study was to determine the relationship between C. sputorum subsp. mucosalis and C. sputorum subsp. sputorum, C. sputorum subsp. bubulus and reference strains of other recognized Campylobacter species by means of deoxyribonucleic acid (DNA) hybridization experiments.

Materials and methods

Organisms

The origin of all of the strains used in this study are listed in Table 1. In all but three cases the type strains were included. In the case of C. sputorum subsp. sputorum, the type strain ER-33 (10) is no longer extant; therefore, VPI strain S-17 was used as the reference strain in this study. This strain fits the original description of the species (10) and has been used by other investigators as a reference strain for C. sputorum subsp. sputorum (5,22). In the cases of C. "hyointestinalis" (3) and C. "fecalis" (2) type strains have not been designated and the strains used were the reference strains suggested by the authors who proposed these names.

Growth conditions

Stock cultures of C. sputorum subsp. mucosalis and C. concisus were maintained in semisolid Brucella medium supplemented with 0.3% fumaric acid and 0.16% agar (semisolid BF medium), adjusted to pH 7.0 with KOH. Cultures were incubated under an atmosphere of 6% O_2, 5% CO_2, 15% H_2 and 74% N_2. Stock cultures of other campylobacters were maintained in semisolid Brucella medium (0.16% agar) incubated aerobically. Semisolid Brucella medium was supplemented with 1.0% NaCl for growth of C. nitrofigilis (14). All stock cultures were incubated at 37°C with the exception of C. nitrofigilis and the aerotolerant campylobacters (16), which were incubated at 30°C. Stock cultures were transfered weekly and also stored in liquid nitrogen.

For DNA isolation, C. mucosalis strains were inoculated into semisolid BF medium and incubated at 37°C under an atmosphere of 6% O_2, 5% CO_2, 15% H_2 and 74% N_2. After 48 h, the top 1-2 ml of growth from two tubes was used to inoculate a Roux bottle containing a diphasic medium [200 ml of BFF medium (BF medium supplemented with 0.2% sodium formate) solidified with 2.5% agar and overlayed with 50 ml of BFF broth] . These cultures were incubated aerobically at 37°C for 24 to 48 h. The growth from eight Roux bottles was usually sufficient to yield enough DNA for the experiments. Strain CG-1, from C. Gebhart, which is an unclassified, catalase-negative or weakly catalase-positive strain (CNW) strain, similar to those described by Sandstedt et al. (19) was grown under similar conditions; however, FBP (ferrous-bisulfite-pyruvate) medium (4) was used in the Roux bottles instead of BFF. C. sputorum strains were grown in Roux bottles containing BF medium as described above and the growth from 2 bottles was used to inoculate 1 liter of BF broth in a 2-liter Erlenmeyer flask. These cultures were incubated aerobically with shaking at 37°C for 24 h. Growth conditions for the catalase-positive campylobacters, with the exception of C. nitrofigilis were described in a previous report (18). C. nitrofigilis was grown in the same way as the other catalase-positive strains, except that all media were supplemented with 1.0% NaCl (14). All cultures were checked for purity by phase-contrast microscopy prior to harvesting.

DNA isolation, DNA homology experiments and determination of DNA base composition

DNA was extracted and purified by the hydroxylapatite (HA) procedure described by Johnson (6). The S-1 nuclease procedure described by Johnson (6) was used for the DNA homology experiments and the homology experiments were performed under "optimal" conditions , i.e., 25°C below the thermal melting point (T_m) of the DNA (12). DNA from C. sputorum subspecies mucosalis NCTC 11,000^T was labeled in vitro with ^{125}I according to the method of Selin et al. (20).

The thermal melting point (T_m) of the DNA from C. sputorum subsp. mucosalis strains NCTC 11,000^T and 79-12009 was determined using the method described by Johnson (6) and the mole percent guanine plus cytosine (mol% G + C) was determined using the equation of Mandel et al. (11). DNA from Escherichia coli strain b, which has a mol% G + C of 51, was used as a reference.

Physiological characteristics

The procedures for determining growth in 1% Oxgall, 3.5% NaCl, 1% glycine and minimal medium (MM); H_2S production in Sulfide-Indole-Motility (SIM) medium, from cysteine (lead acetate strips) and on Triple Sugar Iron (TSI) agar slants; growth at 25 and 42°C and susceptibility to nalidixic acid and cephalothin have been described in a previous report (18). Nitrate and nitrite reduction were tested as previously described (18), with the exception that the final concentration of KNO_3 in the medium was 0.7% instead of 1.0%. Deoxyribonuclease (DNase) activity and the ability to grow on 1.5% NaCl plates were tested as previously described (18) except that the basal medium was replaced with Brucella agar supplemented with 0.3% fumaric acid,

pH 7.0. In all of the tests listed above, cultures were incubated under an atmosphere of 6% O_2, 5% CO_2 and 15%. H_2 and 74% N_2.

Catalase activity, oxidase activity, hippurate hydrolysis and anaerobic growth in 0.1% trimethylamine-N-oxide (TMAO) were tested as previously described (18).

Results and Discussion

All seven strains of C. sputorum subsp. mucosalis used in this study showed a high level of DNA homology (≥92%) with the type strain of C. sputorum subsp. mucosalis (NCTC 11,000^T) but no significant homology with any of the other reference strains tested, including the reference strain of C. sputorum subsp. sputorum (S-17) and the type strain of C. sputorum subsp. bubulus (ATCC 33562^T) (Table 2).

The base composition of the DNA from C. sputorum subsp. mucosalis strains 79-12009 and NCTC 11,000^T was 38 and 39 mol % G + C, respectively, which is higher than the value of 34 mol% G + C reported for NCTC 11,000^T by Lawson et al. (8). The difference may be attributable in part to the use of different equations for calculating the base composition of the DNA. The Mandel equation (11) was used for determining the values given in this paper, while the Marmur equation (13) was used by Lawson et al. When the mol% G + C values for strains 79-12009 and NCTC 11,000^T were recalculated using the Marmur equation, values of 36 and 37 were obtained, which is still higher than the value reported by Lawson et al. (8). The reason for the discrepancy is unknown; however, our results are consistent with the value of 38 mol% G + C for NCTC 11,000^T obtained by Owen and Leaper (17) using the bouyant density method.

C. sputorum subsp. mucosalis strains can be distinguished from all other catalase-negative Campylobacter species except C. concisus (22) by their requirement for H_2 or formate for microaerophilic growth and H_2 and fumarate or formate and fumarate for anaerobic growth (21). Although C.

concisus strains are similar in their phenotypic characteristics to C. sputorum subsp. mucosalis (21,22), they show only a very low level of DNA homology with C. sputorum subspecies mucosalis NCTC 11,000^T (Table 2). C. sputorum subsp. mucosalis strains can be distinguished from C. concisus strains by their susceptibility to cephalothin, ability to grow at 25°C (Roop, R. M. II, R. M. Smibert, J. L. Johnson and N. R. Krieg, "DNA homology studies of the catalase-negative campylobacters and an emended description of Campylobacter sputorum, Canadian Journal of Microbiology, manuscript submitted) and by the "dirty yellow" color of the colonies (21). The color is best seen when a colony is picked and smeared on a piece of white paper (9).

Growth in a medium containing 1.5% NaCl and the inability to grow in the presence of 1% glycine were reported by Lawson et al. (8) to be characteristics of C. mucosalis. During the course of the study reported on in this paper, we found that all 8 of the strains tested, including the type strain (NCTC 11,000^T) were able to grow in the presence of 1% glycine. We perform this test in a semisolid medium, which is probably the method that is used in most diagnostic laboratories, owing to its ease of inoculation and easy interpretation of results. Lawson et al. (9) inoculated agar plates containing 1% glycine with a velour pad that had been charged with inocula from a blood agar plate. They also used a shorter incubation period (24 to 48 h), compared with the 4-to-5 day incubation we used. In addition, we found that only 3 of 8 of the C. mucosalis strains used in our study grew on 1.5% NaCl agar plates and those grew very scantily. This might be due to differences in the basal media or the methodology used for inoculating the plates. Oxoid blood agar base No. 2 (CM 271) was used as the basal medium by Lawson et al. (9),

whereas BF agar was used in our study. The NaCl agar plates used in our study were inoculated by continuous streaking with a loop needle with inocula from a 24- to 48-h-old semi-solid culture, whereas Lawson et al. (9) inoculated NaCl agar plates in the same manner as their glycine agar plates. We believe that our method is the more sensitive of the two for determining the ability to grow in 1.5% NaCl. For instance, growth in 1.5% NaCl is an important differential characteristic for C. laridis (1,18) and those strains which we have tested grow well using our method.

The description of C. sputorum subsp. mucosalis given in Bergey's Manual of Systematic Bacteriology (21) is based on earlier descriptions of this organism (7,8) and does not reflect the discrepancies with the original description that were found during the course of the present study. The results of our DNA homology experiments (Table 2) indicate that C. sputorum subsp. mucosalis is a distinct species and is not a subspecies of C. sputorum. We propose, therefore, that the name C. sputorum subsp. mucosalis be changed to C. mucosalis sp. nov., comb. nov., and we provide the following emended description.

Description of Campylobacter mucosalis (Lawson, Leaver, Pettigrew and Rowland, 1981) sp. nov., comb. nov. The following description of Campylobacter mucosalis (mu. co'. sal. is. L. n. mucosus, mucus, L. f. adj. mucosal, L. gen. n. mucosalis, of mucus, pertaining to mucus) is based upon descriptions given by Lawson et al. (8,9) and Smibert (21) and upon observations made during the course of this study. Short, irregularly curved, Gram-negative cells, that may appear as spiral forms, 0.25-0.3 μm in diameter and 1-3 μm in length. Motile by means of a single polar flagellum. Coccoid

bodies and filamentous forms may be seen in older cultures. Colonies are 1.5 mm in diameter, circular, raised with a flat surface. The colonies have a dirty yellow color that is best seen by smearing the colony on a piece of white paper (9). Requires either hydrogen or formate as an electron donor for growth. Grows under microaerophilic conditions (6% O_2, 5% CO_2, 15% H_2 and 74% N_2) where O_2 serves as the electron acceptor, or anaerobically with fumarate as the terminal electron acceptor. Contains appreciable amounts of lauric acid (C_{12}) in its cellular fatty acids (15). Other phenotypic characteristics of the type strain (NCTC 11,000^T) and 7 other strains are listed in Table 3.

Isolated from the intestinal mucosa of pigs with porcine intestinal adenomatosis, necrotic enteritis, regional ileitis and proliferative hemorrhagic enteropathy; also isolated from the oral cavity of pigs.

The guanine plus cytosine (G + C) content of the DNA from the type strain, NCTC 11,000^T, is 39 mol%, calculated by the Mandel equation (11). The thermal melting point (T_m) of the DNA is 84.3°C in 1X SSC buffer (0.15 M NaCl, 0.015 M Na citrate, pH 7.0).

C. mucosalis NCTC 11,000^T shows less than 9% DNA homology with reference strains of C. sputorum subsp. sputorum, C. sputorum subsp. bubulus, C. concisus, C. fetus subsp. fetus, C. fetus subsp. venerealis, C. jejuni, C. coli, C. laridis, C. nitrofigilis, C. "fecalis", C. "hyointestinalis", CNW strains and aerotolerant campylobacters (Table 2).

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TABLE 1. Bacterial strains used in this study.

Species and strain	Source	Origin
C. sputorum subsp. *mucosalis*:		
NCTC 11,000^T	G. H. K. Lawson, Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Midlothian, Scotland	Porcine
79-12009	G. E. Ward, Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, Minnesota	Porcine
P-9681C	J. Hayes, Purdue University School of Veterinary Medicine, West Lafayette, Indiana	Porcine
P-10546C	J. Hayes	Porcine
P-5007	J. Hayes	Porcine
P-11508	J. Hayes	Porcine
P-5494	J. Hayes	Porcine
P-10742	J. Hayes	Porcine
C. sputorum subsp. *sputorum*:		
S-17	R. M. Smibert, Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia	Human
C. sputurom subsp. *bubulus*:		
ATCC 33562^T	American Type Culture Collection	Bovine

Table 1, continued.....

Species and strain	Source	Origin
C. "fecalis":		
11363[a] (ATCC 33709)	B. D. Firehammer, Veterinary Research Laboratory, Montana State University, Bozeman, Montana	Ovine
C. concisus:		
ATCC 33237[T]	A. C. R. Tanner, Forsyth Dental Center, Boston, Massacuesetts	Human
C. fetus subsp. fetus:		
ATCC 27374[T]	American Type Culture Collection	Ovine
C. fetus subsp. venerealis:		
ATCC 19438[T]	American Type Culture Collection	Bovine
C. jejuni:		
ATCC 33560[T]	American Type Culture Collection	Bovine
C. coli:		
ATCC 33559[T]	American Type Culture Collection	Porcine

Table 1, continued.....

Species and strain	Source	Origin
C. laridis:		
NCTC 11352[T]	National Collection of Type Cultures, London	Avian
C. "hyointestinalis":		
80-4577-4[b] (ATCC 35217)	C. Gebhart, Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, Minnesota	Porcine
C. nitrofigilis:		
ATCC 33309[T]	American Type Culture Collection	Spartina alterniflora roots
CNW strains:		
CG-1	C. Gebhart	Canine
Aerotolerant campylobacters:		
02790	S. D. Neill, Veterinary Research Laboratory, Belfast, Ireland	Porcine

[a]Suggested type strain (B. D. Firehammer, personal communication).

[b]Suggested type strain (C. Gebhart, personal communication).

TABLE 2. DNA homology of *C. sputorum* subsp. *mucosalis* strains and campylobacter reference strains with *C. sputorum* subsp. *mucosalis* NCTC 11,000^T.

Unlabeled DNA from strain	% homology with DNA from *C. sputorum* subsp. *mucosalis* NCTC 11,000^T
C. sputorum* subsp. *mucosalis	
NCTC 11,000^T	100
P5007	96
P5494	96
P11508	96
P10546C	95
P9681C	92
P10742	92
79-12009	92
C. sputorum* subsp. *sputorum	
S-17	3
C. sputorum* subsp. *bubulus	
ATCC 33562^T	5
C. "fecalis"	
11363	3
C. concisus	
ATCC 33237^T	9
C. fetus* subsp. *fetus	
ATCC 27374^T	4
C. fetus* subsp. *venerealis	
ATCC 19438^T	4

Table 2, continued.....

Unlabeled DNA from strain	% homology with DNA from *C. sputorum* subsp. *mucosalis* NCTC 11,000^T
C. "hyointestinalis"	
80-4577-4	5
C. jejuni	
ATCC 33560^T	6
C. coli	
ATCC 33559^T	2
C. laridis	
NCTC 11352^T	2
C. nitrofigilis	
ATCC 33309^T	2
aerotolerant campylobacters	
02790	2
CNW strains	
CG-1	2

TABLE 3. Phenotypic characteristics of *Campylobacter mucosalis*[a].

Characteristic	Reaction	
	NCTC 11,000[T]	7 other strains
Catalase	-	-
Oxidase	+	+
Nitrate reduction	+	+[b]
Nitrite reduction	-	d[b]
Growth in:		
1% oxgall	+	+
1.5% NaCl (plates)	-	-
3.5% NaCl	-	-
1% glycine	+	+
minimal medium[c]	-	-
Anaerobic growth in 0.1% TMAO	-	-
H_2S production:		
SIM medium	+	+
Lead acetate strips	+	+
Triple Sugar Iron (TSI) agar	+	+
Growth at:		
25°C	+	+
42°C	+	+
Hippurate hydrolysis	-	-
DNase activity	-	[-]
Susceptibility to:		
nalidixic acid, 30 µg disk	+	-
cephalothin, 30 µg disk	+	+

[a]Symbols: + = 90-100% of strains positive, d = 26-75% of strains positive, [-] = 11-25% of strains positive, - = 0-10% of strains positive.

[b]Lawson et al. (7,8,9) reported that *C. mucosalis* strains reduced nitrite; however, they used a different basal medium (nitrate broth supplemented with 5% inactivated horse serum) than was used in the study reported here (Brucella medium).

Table 3, continued.....

[c]Roop et al. (18).

Summary and Conclusions

DNA homology experiments showed that the 108 campylobacter strains used during the course of this study could be placed into 10 distinct DNA homology groups (species) corresponding to C. fetus, C. "hyointestinalis", C. jejuni, C. coli, C. laridis, C. sputorum, C. mucosalis, C. concisus, and two unnamed groups currently known as the aerotolerant campylobacters and the CNW strains. The campylobacter strains used in this study were also characterized phenotypically, and the characteristics that provided the most reliable identification of these strains at the species, subspecies and biovar levels were growth at 25 and 42°C, sensitivity to nalidixic acid and cephalothin, growth in a semisolid medium containing 1% glycine, 1% oxgall or 3.5% NaCl, growth on agar plates containing 1.5% NaCl, growth in a semisolid minimal medium (MM), anaerobic growth in 0.1% trimethylamine-N-oxide (TMAO), H_2S production in Sulfide-Indole-Motility (SIM) medium, or on triple sugar iron (TSI) agar slants, hippurate hydrolysis, aerobic growth on agar plates, a requirement for H_2 or formate for microaerophilic growth and H_2 and fumarate or formate and fumarate for anaerobic growth, alkaline phosphatase activity and deoxyribonuclease (DNase) activity.

C. fetus subsp. fetus and C. fetus subsp. venerealis cannot be distinguished by DNA homology. However, the subspecies designations were maintained for practical reasons. C. fetus subsp. fetus and C. fetus subsp. venerealis cause two distinctly different types of fertility problems in animals and the methods used for treating infected animals depends on which subspecies of C. fetus is involved. Also, C. fetus subsp. fetus can be pathogenic for humans, but C. fetus subsp. venerealis is not. These two organisms can be differentiated in the laboratory by the ability of C. fetus

subsp. fetus to grow in the presence of 1% glycine. At present, this is the only laboratory test available for distinguishing between these organisms.

Although C. "hyointestinalis" has many phenotypic characteristics in common with C. fetus, C. "hyointestinalis" is not related to C. fetus at the species level. C. "hyointestinalis" has been isolated from the lesions of proliferative ileitis in pigs and from the feces of calves. C. "hyointestinalis" can be distinguished from C. fetus by the ability of C. "hyointestinalis" to produce H_2S on a TSI slant. Further studies on these organisms have indicated that H_2 is required in the incubation atmosphere in order to get consistent production of H_2S on TSI slants (see Appendix A).

Although C. jejuni and C. coli strains are very similar in their phenotypic characteristics and are more closely related to each other by DNA homology than they are to any of the other Campylobacter species, C. jejuni and C. coli strains represent distinct species. Because C. jejuni and C. coli appear to occupy different ecological niches, the proper identification of these strains to the species level is very important in the study of the epidemiology of campylobacter-associated gastroenteritis in humans. C. jejuni strains can be differentiated from C. coli strains by their ability to hydrolyze hippurate, their inability to grow in a minimal medium (MM) and their failure to produce H_2 on TSI slants. Prior to this study, the hippurate hydrolysis test was the only reliable test available for differentiating between C. jejuni and C. coli. Biotyping schemes were also developed for C. jejuni and C. coli, which should be especially useful for small clinical laboratories that do not have access to the reagents or equipment required for more sophisticated epidemiological studies.

C. laridis strains resemble C. jejuni and C. coli in many of their phenotypic characteristics, but they are resistant to nalidixic acid. For this reason they were formerly referred to as the NARTC (nalidixic acid-resistant thermophilic Campylobacter) strains. The majority of the isolations reported for these strains have been from seagulls of the genus Larus, from which the name laridis was derived. The importance of these organisms in animal or human disease is unknown at the present. However, we did confirm by means of DNA homology experiments that a strain of C. laridis had been isolated from the blood of an immunocompromised patient suffering from septicemia. This was the first recorded case of a strain of C. laridis causing disease in humans or animals. C. laridis strains can be differentiated from C. jejuni and C. coli strains by their ability to grow anaerobically in the presence of 0.1% trimethylamine-N-oxide (TMAO), growth on agar plates containing 1.5% NaCl and by their resistance to nalidixic acid.

C. sputorum contains the organisms formerly known as C. sputorum subsp. sputorum, C. sputorum subsp. bubulus and C. "fecalis". Because there appears to be a great deal of host specificity within phenotypically distinct groups within this species, we have proposed that C. sputorum be divided into 3 biovars. C. sputorum biovar sputorum (formerly known as C. sputorum subsp. sputorum) is part of the normal flora of the human mouth and can occasionally be isolated from normal human feces. It is not believed to be pathogenic, however, two of the three biovar sputorum strains that we studied were isolated from human clinical samples. C. sputorum biovar bubulus (formerly (C. sputorum subsp. bubulus) is part of the normal flora of the bovine reproductive tract and is not considered to be a pathogen. C. sputorum

biovar fecalis (formerly C. "fecalis") is a commensal isolated from sheep feces. All three of these organisms produce abundant H_2S on SIM and TSI media. C. sputorum biovar sputorum can be distinguished from biovars bubulus and fecalis by the ability of biovar sputorum strains to grow in the presence of 1% oxgall and their inability to grow in 3.5% NaCl. C. sputorum biovar fecalis strains are identical in their phenotypic characteristics to C. sputorum biovar bubulus strains with the exception that biovar fecalis strains are catalase positive.

C. mucosalis and C. concisus strains are unique among the currently recognized Campylobacter species because they require either H_2 or formate as an electron donor for growth. C. mucosalis strains were formerly classified as C. sputorum subsp. mucosalis. However, DNA homology studies showed that these strains formed their own distinct DNA homology group and were not related at the species level to any of the other Campylobacter species tested, including C. sputorum and C. concisus. C. mucosalis is isolated from lesions of porcine intestinal adenomatosis, which is also known as porcine proliferative ileitis. It is believed to play a role in this disease, either alone or in conjunction with C. "hyointestinalis" (see above). C. concisus has been isolated from the mouths of humans with periodontal disease, but the role of C. concisus in periodontal disease, if any, is not known. C. mucosalis strains can be differentiated from C. concisus strains by their sensitivity to cephalothin and ability to grow at 25°C.

The are two groups of campylobacters that are currently unnamed and each one represents its own distinct DNA homology group. The aerotolerant campylobacters were originally isolated from porcine and bovine abortions on

Leptospira enrichment medium. These organisms were able to grow aerobically on subculture, a characteristics that is not shared by the other campylobacters. They have since been isolated from mastitis in cattle and from ovine and equine abortions. The "catalase-negative or weak" (CNW) strains are isolated from canine and feline feces. They have some phenotypic characteristics in common with C. coli strains, but are not related at the species level to C. coli or any of the other species of Campylobacter used in this study. The lack of catalase activity and strong alkaline phosphatase activity are a distinguishing combination of characteristics for these organisms.

The study presented here is the most comprehensive of its kind that has been performed on the campylobacters to date. The species relationships have been determined by means of DNA homology experiments and the phenotypic characteristics that provide the most reliable identification of these organisms at the species, subspecies and biovar levels have been presented. In some cases the usefulness of previously described differential tests has been confirmed or rejected, and in some cases, the number of useful differential characteristics has been expanded.

Appendix A

Unpublished Data

C. "hyointestinalis" strains from calves. In December, 1984, I received 5 strains of campylobacters that had been isolated from the feces of healthy calves (Myers et al. 1984) from B. D. Firehammer, Department of Veterinary Science, Montana State University, Bozeman, Montana. These strains had phenotypic characteristics similar to those described for C. "hyointestinalis" (Gebhart et al. 1983), although previously the only reported isolations of C. "hyointestinalis" had been from pigs.

In a limited DNA homology experiment, DNA from Firehammer strain 20,071 was labeled and compared to a number of Campylobacter reference strains including the suggested type strain for C. "hyointestinalis" 80-4577-4. The calf strains were also characterized phenotypically. The results of the DNA homology experiment are shown in Table 1 and the phenotypic characteristics of the calf strains are given in Table 2, along with the phenotypic characteristics for strain 80-4577-4.

The strains from calves showed 83-100% DNA homology with strain 20,071, which in turn, showed 82% DNA homology with the suggested type strain for C. "hyointestinalis" 80-4577-4. These results indicate that C. "hyointestinalis" can also be isolated from the feces of healthy calves. A group from Sweden has reported the isolation of C. "hyointestinalis"-like organisms from cattle suffering from chronic diarrhea (Ursing et al. 1984) and Myers et al. (1984) recently reported finding C. "hyointestinalis"-like bacteria in the feces of normal calves. Some of the latter organisms were used in this study. The calf strains studied here were similar to the C. "hyointestinalis" strains from pigs we had studied earlier, with the exception that 3 of the calf strains would not grow anaerobically in 0.1% TMAO, while all of the porcine

strains did grow with this compound as a terminal electron acceptor. It was also found that in order to get consistent production of H_2S on TSI slants with the calf strains, it was necessary to have some H_2 present in the atmosphere. Other invsetigators have encountered this same situation with H_2 production by C. "hyointestinalis" on TSI slants (C. Gebhart, personal communication).

The results of this short study indicate that C. "hyointestinalis" can be isolated from cattle as well as from pigs, that anaerobic growth in TMAO may not be as useful for identifying these strains as previously thought (see Chapter 1) and that H_2 should be included in the atmosphere when testing C. "hyointestinalis" strains for H_2S production on TSI slants.

C. jejuni strains from rabbits. In April 1984, I received 4 strains suspected of being Campylobacter jejuni that had been isolated from rabbits from Dr. J. H. Bryner of the National Animal Disease Center, Ames, Iowa. For many years, Dr. Bryner has been using a phage typing system for distinguishing C. jejuni from C. coli strains (J. H. Bryner, personal communication). Although the 4 strains that he sent were positive for hippurate hydrolysis, they were lysed by the C. coli phage. He was curious as to whether these strains were C. jejuni strains that were attacked by the C. coli phage, or hippurate-positive C. coli strains.

Using labeled DNA from Bryner strain 1684, DNA homology experiments showed that all 4 of the rabbit strains were highly related (94-100% DNA homology) and were related at the species level to two C. jejuni reference strains, the type strain, ATCC 33560 and strain H840. Reference strains

representing other Campylobacter species were also used in this experiment (Table 3).

The 4 C. jejuni strains had the phenotypic characteristics that are distinctive for C. jejuni strains (Table 4). In fact, these strains were the most strongly hippurate-positive strains that I have yet encountered, the reaction mixture turning a dark purple immediately after adding the ninhydrin. They would not grow in MM and did not produce any H_2S on TSI slants.

The results of this brief study indicate that speciation of suspected C. jejuni or C. coli strains by means of phage typing may result in a mistaken identification at the species level, as has been demonstrated for speciation by serotyping for these organisms (Hébert et al. 1984). This further proves that although serotyping and phage typing are very useful for epidemiological purposes, these procedures are most useful when the organisms in question have first been correctly identified to the species level.

Nitrogen-fixing campylobacter. In 1980, McClung and Patriquin reported isolating a small, curved, Gram-negative, nitrogen-fixing bacterium from the roots of a salt marsh grass, Spartina alterniflora Loisel. This organism was obligately microaerophilic, did not attack sugars, utilized TCA cycle intermediates and amino acids as carbon sources, was highly motile with a "corkscrew" type of motility and its DNA had a mol% G + C of 28. For these reasons, they placed this organism in the genus Campylobacter and gave it the name C. nitrofigilis (McClung et al. 1983). Besides its ability to fix nitrogen, this organism differs from other campylobacters in its ability to hydrolyze urea, to produce a pigment from tryptophan and by its requirement

for at least 0.5% NaCl in the medium (McClung et al. 1983). In addition, this organism was isolated from a symbiotic association with a plant, while the other Campylobacter species are either pathogens or commensals of animals.

With these differences in mind, we obtained the type strain of C. nitrofigilis ATCC 33309 from the American Type Culture Collection, and performed a DNA homology experiment using labeled DNA from ATCC 33309 as a reference and unlabeled DNA from a number of reference strains representing other Campylobacter species. Table 5 shows that C. nitrofigilis did not show any significant DNA homology with any of the other campylobacter reference strains. Although the results of DNA homology studies alone are not sufficient grounds for excluding this organism from the genus Campylobacter, the lack of significant DNA homology combined with the unique phenotypic characteristics of this organism imply that this organism is probably not a Campylobacter species. However, only rRNA:DNA hybridization studies will provide a definite answer to this problem.

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Table 1. DNA homology of campylobacter reference strains with Firehammer strain 20,071.

Strain	% homology with DNA from strain 20,071
Firehammer's calf strains	
20,071	100
20,049	93
20,064	84
20,048	90
19922-1	83
"C. hyointestinalis"	
80-4577-4ST	82
81-14151-1	85
C. fetus subsp. fetus	
ATCC 27374^{T}	21
C. fetus subsp. venerealis	
ATCC 19438^{T}	23
C. jejuni	
ATCC 33560^{T}	3
H840	2

Table 1, continued.....

Strain	% homology with DNA from strain 20,071
C. coli	
ATCC 33559^{T}	2
541	2
C. laridis	
NCTC 11352^{T}	3
aerotolerant campylobacters	
02790	2
C. nitrofigilis	
ATCC 33309^{T}	1
C. sputorum	
S-17^{NT} (biovar sputorum)	4
ATCC 33562 (biovar bubulus)	4
11363 (biovar fecalis)	5
C. mucosalis	
NCTC 11,000^{T}	4

Table 1, continued.....

Strain	% homology with DNA from strain 20,071
CNW strain	
CG-1	2

[a]Superscripts: T = type strain, NT = proposed neotype strain, ST = suggested type strain.

Table 2. Phenotypic characteristics of Firehammer's C. "hyointestinalis" strains from calves and the suggested type strain 80-4577-4.

Characteristic	Strain 20,071	20,049	20,064	19922-1	20,048	80-4577-4
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+
Nitrite reduction	-	-	-	-	-	-
H_2S producton:						
SIM medium	-	-	-	-	-	-
TSI slant[a]	+	+	+	+	+	+
Pb acetate strip	+	+	+	+	+	+
Growth at:						
25°C	-	+	-	-	-	+
42°C	+	+	+	+	+	+
Growth in:						
1% glycine	+	+	+	+	+	+
1% oxgall	+	+	+	+	+	+
3.5% NaCl	-	-	-	-	-	-
Anaerobic growth in 0.1% TMAO	+	-	-	+	-	+
Hippurate hydrolysis	-	-	-	-	-	-
Sensitivity to:						
nalidixic acid	-	-	-	-	-	-
cephalothin	+	+	+	+	+	+

Table 2, continued.....

[a] H_2 is required in the incubation atmosphere for consistent results.

Table 3. DNA homology values for Bryner's rabbit strains and campylobacter reference strains with Bryner strain 1684.

Species and Strain	% DNA homology with strain 1684
Bryner's rabbit strains	
1684	100
1687	99
1590	97
1591	94
C. jejuni	
ATCC 33560^T	85
H840	82
C. coli	
ATCC 33559^T	38
C. fetus subsp. *fetus*	
ATCC 27374^T	14
C. fetus subsp. *venerealis*	
ATCC 19438^T	4

Table 3, continued.....

Species and Strain	% DNA homology with strain 1684
C. "hyointestinalis"	
80-4577-4[ST]	4
C. laridis	
NCTC 11352[T]	13
C. sputorum	
S-17[NT] (biovar sputorum)	4
ATCC 33562 (biovar bubulus)	3
11363 (biovar fecalis)	4
C. mucosalis	
NCTC 11,000[T]	3
CNW strains	
CG-1	12
C. nitrofigilis	
ATCC 33309[T]	3

Table 3, continued.....

Species and Strain	% DNA homology with strain 1684
C. concisus	
13086[T]	2
aerotolerant campylobacters	
02790	4

[a]Superscripts: T = type strain, NT = proposed neotype strain, ST = suggested type strain.

Table 4. Phenotypic characteristics of Bryner's C. jejuni strains from rabbits and the type strain of C. jejuni ATCC 33560.

Characteristic	Strain 1684	1687	1590	1591	ATCC 33560
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Nitrite reduction	+	+	+	+	+
H_2S production:					
SIM medium	-	-	-	-	-
TSI slants	-	-	-	-	-
Growth in:					
1% glycine	+	+	+	+	+
1% oxgall	+	+	+	+	+
3.5% NaCl	-	-	-	-	-
minimal medium	-	-	-	-	-
Growth at:					
25°C	-	-	-	-	-
42°C	+	+	+	+	+
Hippurate hydrolyis	+	+	+	+	+
DNase activity	+	+	+	+	-
Sensitivity to:					
nalidixic acid	+	+	+	+	+
cephalothin	-	-	-	-	-

Table 5. DNA homology of *C. nitrofigilis* ATCC 33309 with reference strains of other *Campylobacter* species.[a]

Species and strain	% homology with DNA from strain ATCC 33309
C. nitrofigilis	
ATCC 33309[T]	100
C. fetus subsp. *fetus*	
ATCC 27374[T]	1
C. fetus subsp. *venerealis*	
ATCC 19438[T]	1
C. jejuni	
ATCC 33560[T]	2
C. coli	
ATCC 33559[T]	0
C. laridis	
NCTC 11352[T]	0

Table 5, continued.....

Species and strain	% homology with DNA from strain ATCC 33309
C. "hyointestinalis"	
80-4577-4[ST]	0
C. sputorum	
S-17[NT] (biovar sputorum)	1
ATCC 33562 (biovar bubulus)	1
11363 (biovar fecalis)	0
C. mucosalis	
NCTC 11,000[T]	4
Aerotolerant campylobacters	
02790	6
CNW strains	
CG-1	0

[a]Superscripts: T = type strain, NT = proposed neotype strain, ST = suggested type strain.

Appendix B

Materials and Methods

Culture media

The following culture media were used during the course of this study:

1. Brucella broth and agar.

2. FBP broth (George et al. 1978): Brucella broth supplemented with 0.025% sodium bisulfite, 0.05% ferrous sulfate ($7H_2O$) and 0.05% sodium pyruvate.

3. FBP agar (George et al. 1978): Brucella agar supplemented with 0.025% each of sodium bisulfite, ferrous sulfate ($7H_2O$) and sodium pyruvate for use in plates.

4. MFBP agar: FBP agar supplemented with an additional 1.0% agar for use in Roux bottles.

5. BF broth: Brucella broth supplemented with 0.3% fumaric acid and adjusted to pH 7.0 with KOH pellets.

6. BF agar: BF broth supplemented with 1.5% agar for use in plates.

7. MBF agar: BF broth supplemented with 2.5% agar for use in Roux bottles.

8. BFF broth: Brucella broth supplemented with 0.3% fumaric acid, 0.2% sodium formate and adjusted to pH 7.0 with KOH pellets.

9. MBFF agar: BFF broth supplemented with 2.5% agar for use in Roux bottles.

10. BS broth: Brucella broth supplemented with 1.0% NaCl.

11. BS agar: BS broth supplemented with 1.5% agar for plates.

12. MBS agar: BS broth supplemented with 2.5% agar for Roux bottles.

In some cases, Brucella medium was made from scratch and the following formulation was used:

Ingredient	Grams per liter
Bacto-Tryptone (Difco)	10
Bacto-Peptone (Difco)	10
Yeast Extract (BBL)	2
NaCl	5
Sodium Citrate	1

Late in the study, a good deal of Tryptose phosphate broth (approximately 10 lb.) was offered free of charge and with the addition of 2 g per liter of yeast extract was used as a substitute for Brucella medium except in cases where cells were being grown for the alkaline phosphatase assay.

The formulation of Tryptose phosphate medium is as follows:

Ingredient	Grams per liter
Bacto-Tryptose (Difco)	20
Dextrose	2
NaCl	5
Na_2HPO_4	2.5

Maintenance and preservation of cultures

Cultures were maintained in 10 ml of Brucella broth supplemented with 0.16% agar (semisolid Brucella medium) in 20 x 125 mm screw-capped tubes at 37°C (30°C for the aerotolerant campylobacter strains and C. nitrofigilis) and transferred weekly with sterile Pasteur pipets. Semisolid BS was used for the

maintenance of C. nitrofigilis and semisolid BF medium for the maintenance of C. mucosalis and C. concisus strains. C. mucosalis and C. concisus cultures were maintained under an atmosphere of 6% O_2, 5% CO_2 ,15% H_2 and 74% N_2. All other strains were maintained aerobically.

Type strains, reference strains and strains having unusual characteristics were stored in liquid nitrogen. Strains to be stored in liquid nitrogen were grown for 24-48 h in semisolid Brucella medium. The top 1-2 ml of growth from this culture was resuspended in 2.0 ml of nutrient broth containing 10% sterile dimethyl sulfoxide (DMSO). Approximately 0.5 ml of this mixture was pipetted into either a screw-capped disposable serum tube (Vanguard N-1076) or a sterile, cotton-stoppered 1 ml glass cryule (Wheaton 200) and placed in liquid N_2.

Growth of cultures for DNA extraction

Originally all of the catalase-positive strains used in this study, with the exception of C. sputorum biovar fecalis and C. nitrofigilis, were grown in FBP medium as described in Chapter 1. However, it was later found that the method given below is better for growing large volumes of all of the catalase-positive campylobacters with the exception of the aerotolerant strains and C. nitrofigilis. This method also worked best for strains of C. sputorum biovars sputorum and bubulus.

Two hundred ml of MBF agar was poured into a Roux bottle (Corning 1290) and the bottle capped with a double layer of aluminum foil. The bottles were autoclaved for 30 min in an upright position and layed flat to cool. For each bottle, 50 ml of BF broth was prepared in a cotton-stoppered 250 ml

flask and sterilized by autoclaving for 20 min and used as the liquid overlay. Before inoculation, the BF broth was aseptically added to the Roux bottle and the bottle capped with a sterile #5 rubber stopper through which a piece of glass tubing, approximately 4 to 5 inches in length and stuffed with cotton had been inserted. The stoppers were sterilized separately just prior to use.

Each Roux bottle was inoculated with the top 1-2 ml from 2 24-48 h old cultures grown in semisolid Brucella medium. Inoculations were performed with sterile 5 ml pipets. After inoculation, the bottles were incubated at 37°C until the broth in the bottles was fairly turbid, usually 24 h. The growth from 2 Roux bottles was aseptically added to 1000 ml of BF broth in a 2 liter Erlenmeyer flask. The 2 liter flasks were incubated at 37°C in a Psychrotherm (New Brunswick Scientific Co., Inc., New Brunswick, N. J.) shaker incubator and shaken at the lowest possible speed or grown in a 37°C waterbath and shaken at a setting of 60 rpm for 12 to 24 h before harvesting.

The aerotolerant campylobacters were grown as above, with the exception that unsupplemented Brucella medium was used throughout, and all incubations were at 30°C. C. nitrofigilis was grown in the same manner as the aerotolerant campylobacters with the exception that BS broth and MBS agar were used.

The CNW strains were grown in Roux bottles as described above, but FBP was used as the basal medium. Attempts to cultures large quantities of these organisms in flasks were unsuccessful, so cells for DNA isolation were isolated directly from Roux bottles. Eight Roux bottles usually yielded enough DNA for the homology experiments. The contents of each bottle was checked by phase microscopy before pooling prior to DNA extraction.

C. mucosalis and C. concisus require either H_2 or formate as an electron donor for microaerophilic growth. Attempts to grow these organisms in flasks containing BFF broth were unsuccessful. Thus, the strains were grown in and directly harvested from Roux bottles in the same manner as the CNW strains, but the inocula for the Roux bottles were grown under an atmosphere of 6% O_2, 5% CO_2, 15% H_2 and 74% H_2 and the medium used in the Roux bottles was MBFF agar and BFF broth.

Buffers and reagents used for DNA isolation

The following reagents and buffers were used in the DNA isolation procedures.

Saline-EDTA buffer: 0.15 M NaCl, 0.01 M Na EDTA, pH 8.0.

Standard Saline Citrate (SSC) buffer: 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0. This buffer is used in several different concentrations, i. e. 1X SSC, 0.1X SSC. A stock solution of 20X SSC was prepared and adjusted to pH 7.0. Other working concentrations were prepared from this stock solution. After dilution, the pH of each of the working buffers was checked and adjusted to pH 7.0 if necessary.

3 M Na acetate, pH 6.0

Chloroform: phenol mixture: 490 ml saline-EDTA saturated liquified phenol, 490 ml chloroform and 20 ml octanol with 8-hydroxyquinoline added to a final concentration of 0.1%. The saline-EDTA saturated phenol was prepared by placing approximately 800 ml of liquified phenol in a glass beaker containing a magnetic stirring bar. The beaker was placed on a magnetic stirring plate

under a fume hood and saline-EDTA buffer added slowly until no more would go into solution.

All of the reagents and buffers listed above were stored in a refrigerator at 4°C in screw-capped 1 liter bottles, with the exception of the chloroform:phenol mixture, which was stored in a 2 liter reagent bottle with a ground-glass stopper.

1 M phoshate buffer: 1 M NaH_2PO_4 and Na_2HPO_4. Prepared by mixing equal volumes of 1 M NaH_2PO_4 and 1 M Na_2HPO_4. The pH of a 1:10 dilution of this buffer was approximately 6.8, but the concentrated stock solution was somewhat higher. Stored in 1 liter screw-capped bottles at room temperature. A few drops of chloroform were added periodically to prevent fungal growth. Refrigeration caused the concentrated phosphate solution to precipitate, however, the 0.1 M phosphate buffer was stored in a refrigerator at 4°C.

20% (w/v) Sodium dodecyl sulfate (SDS): standard reagent grade SDS was used for the DNA isolations. Stored in a screw-capped bottle at room temperature. In cold weather, the bottle was stored in a 37°C incubator.

95% Ethanol: Stored at -20°C in 1 liter screw-capped bottles.

Ribonuclease A, bovine pancreatic (Sigma): 100 mg was dissolved in 100 ml of 0.1X SSC and heated in a hot water bath at 80°C for 20 min to inactivate any residual DNase activity. One hundred (100) ml of cold 0.1X SSC was added and the mixture chilled on ice. The mixture was then placed into 16 x 125 mm screw-capped tube in 10 ml amounts and stored until needed at -20°C.

Ribonuclease T_1, from Aspergillus oryzae (Sigma): 5000 units of RNase T_1 was dissolved in 10 ml of 0.1X SSC-10^{-2}M MES buffer. This mixture was heated as

above to remove any DNase activity and 10 ml of 0.1X SSC-10^{-2} MES was added and the mixture chilled on ice. Ten ml amounts of RNase T_1 were stored at -20°C in 16 x 125 mm screw-capped test tubes.

DNA Isolation Procedures

The cells were harvested by centrifugation in 250 ml polypropylene centrifuge tubes in a refrigerated centrifuge at 10,000 rpm (9630 x g) for 10 min at 0-to-4°C. The supernatant was carefully poured off into a container of concentrated Lysol. The cell pellets were resuspended in saline-EDTA buffer. The EDTA in this buffer inactivates any DNase activity that might be present when the cells are lysed by binding up Mg^{++}, which is required for DNase activity. It may also make the cells easier to lyse by binding divalent cations that lend strength to the outer membrane of Gram-negative bacteria. The final volume of the cell mixture was adjusted to 25 ml or 50 ml depending on the volume of cells being harvested. The hydroxyapatite (HA) procedure was used to isolate all of the DNA used in the DNA homology experiments. The final volume of the lysing mixture was usually adjusted to 50 ml for the DNA isolations, 25 ml if the yield of cells was low.

One-half ml of RNase A (bovine pancreatic, Sigma) and 0.25 ml of RNase T_1 was added to the cell suspension and mixed well. RNase A is a good general purpose ribonuclease and RNase T_1 is effective against rRNA from organisms having a high mol% G + C content. Next, 20% sodium dodecyl sulfate was added to a final concentration of 1% (2.5 ml to 50 ml, 1.2 ml in 25 ml) and the mixture was swirled under hot running tap water. The SDS disrupts the integrity of the cell membrane because of its detergent action.

Lysis of the cells was indicated by an increase in the viscosity of the lysate and a clearing of the mixture. The amount of viscosity is a good estimate of the amount of DNA present, the greater the viscosity, the more DNA is present. Most of the campylobacters used lysed very easily after the addition of the SDS. The few strains that did not, lysed in a short time if the flask containing the lysate was allowed to sit in a beaker of hot water for a few minutes. Next, the viscosity of the lysate was reduced by brief sonication. This allows the phenol:chloroform used for protein removal to become mixed in more easily and results in shorter strands of double stranded DNA which are more easily adsorbed onto the HA particles. Initially, a Raytheon enclosed sonic oscillation apparatus (Model DF 101) was used and the lysates were subjected to 2 to 3 short bursts of 10 to 15 sec each of sonic oscillation at a setting of 60 to 70% full power.. Later, a probe sonicator became available (Lab Line Ultratip Labsonic System, Lab Line Instruments, Inc., Melrose Park, Illinois) and the lysate was subjected to 2 to 3, 3 to 5 sec intervals of sonication with an intermediate sized tip. After sonication, the proteins were extracted from the lysate with the chloroform:phenol mixture. Fifteen ml of this mixture was added for every 50 ml of cell lysate and the mixture shaken on a wrist-action shaker for 20 min. The mixture was placed in a 50 ml polypropylene centrifuge tube and centrifuged at 12,000 rpm (17,000 x g) for 10 min. The upper (aqueous) layer was carefully removed, using an inverted 10 ml pipet in a propipette and returned to the lysing flask for an second chloroform-phenol extraction. This extraction and centrifugation were performed as before. Care was taken to avoid pulling up the white layer of denatured protein that formed at the interface of the aqueous and the

chloroform-phenol phases. This was especially true after the second phenol extraction. After the second protein extraction, enough 1 M phosphate buffer was added to make the final concentration of the lysate 0.1 M phosphate. The lysate was divided into 25 ml aliquots and 2g (1 tbsp.) of DNA grade hydroxyapatite (Biogel, Biorad) was added to each. This mixture was then shaken on a wrist action shaker for 1 h. The HA-lysate suspension was placed in a 50 ml polypropylene centrifuge tube and centrifuged at top speed in a IEC Model CL clinical centrifuge for 1-2 min to pellet the HA. The supernatant was then poured back into the flask for a second HA elution. The pellet was then washed with 0.1 M PO_4 in the following manner. Eight ml of 0.1 M PO_4 was added to the tube with a Cornwall syringe and the tube was vortexed at a setting of 7 on a Vortex-Genie (Model K-550-G, Scientific Industries, Inc., Springfield, Mass.). Twenty-four ml of 0.1 M PO_4 was added in aliquots of 8 ml each at the interface of the liquid and the tube wall with sufficient force to vigorously mix the HA and the PO_4 buffer. This mixture was centrifuged as before and the supernatant discarded. This washing procedure was repeated 6 times. After the sixth wash, 5 ml of 0.5 M PO_4 was added to the HA pellet and the tube vortexed at the maximum speed on the Vortex-Genie. The tube was placed in the clinical centrifuge and spun as before. The supernatant, which contained the eluted DNA was poured into a 10 ml B-D Multifit glass syringe and filtered through a Whatman glass fiber filter (934-AH, 2.4 cm dia.) in a Gelman 1 in dia. Easy Pressure filter holder. The filtered DNA solution was stored in a 18 x 125 mm screw-capped tube at -20°C until needed. The second HA elution was performed in the same manner described above.

Determination of concentration and purity of DNA preparations

DNA concentration and purity was determined on a Gilford 2400 recording spectrophotometer using the following procedure. A 1:20 dilution of the DNA preparation was made by placing 50 µl of the DNA preparation in 0.95 ml of 0.1X SSC in a 12 x 75 mm serum tube . This mixture was vortexed and 0.25 ml of this mixture placed into a quartz microcuvette (Gilford 1242x103). The OD at 260 nm was determined. The OD of a 1:20 dilution will give the concentration of DNA in mg/ml if the DNA preparation is 100% pure. The purity was determined from the increase in absorbance that occured when the preparation was heated until it became single-stranded. The microcuvette was heated in a thermal cuvette holder (Gilford 2527-1242x82) and the temperature was raised from an original temperature of 50°C at a rate of 1°C per min. The rate was controlled by a thermal progammer (Gilford 2527) and the change in absorbance was plotted on a chart recorder. The purity was determined by dividing the change in OD by the original OD and dividing this value by 0.4. One hundred percent pure DNA should have an increase in OD of 40% when it becomes single stranded. The concentration was then determined by multiplying the OD at 260 nm by the purity.

Preparation of DNA for homology experiments

The first step was to remove the phosphate from the DNA preparations. This was accomplished by dialyzing the preparations against 0.1X SSC. Five to 6 inch strips of dialysis tubing (Spectrapor 2, Fisher, 25 mm dia. 12,000 to 14,000 MW cutoff) were cut and boiled in a solution of 2 to 5% $NaCO_3$ for

several min. The tubing was then washed first in tap water and then with distilled water. The DNA preparations were placed in the dialysis bags and 15 to 20 bags were placed in 4 liter of 0.1X SSC, pH 7.0 in a 4 liter Nalgene beaker with a magnetic stir bar. The beaker was placed on a magnetic stirrer in the cold room (ca. 4°C) and the solution stirred at an intermediate speed for 4 to 6 hours, after which the bags were placed in another 4 liter Nalgene beaker containing fresh dialysis buffer and the dialysis continued with stirring overnight. The next day, the bags were opened with scissors over small glass beakers and the DNA preparations placed into 18 x 125 mm screw-capped tubes and stored at -20°C until needed. Most of the DNA preparations used in the DNA homology experiments were at a concentration of 0.4 mg/ml. Since the yield of DNA from many of the campylobacter strains was lower than this, several preparations from the same strain had to be pooled and concentrated by ethanol precipitation and dissolving in a smaller volume of 0.1X SSC. This was accomplished in the following manner. Several DNA preparations from the same strain, in 0.1X SSC buffer, were combined in one or more 50 ml polypropylene centrifuge tubes and 1/10 volume of 3M sodium acetate added to each tube. The maximum volume in each tube was 13 ml, because 2 volumes of ice-cold ethanol was then added. The tubes were then placed at -20°C for at least 1 hour. The tubes were centrifuged at 17,000 x g for 10 min, the supernatant carefully poured off so that the precipitated DNA was not lost, and the tube inverted and drained. After most of the ethanol was gone, the DNA pellet was resuspended in a small volume of 0.1X SSC, the exact volume depending on the amount of DNA present in the pellet, which could be estimated from the concentrations and purities of the preparations

used. A small amount of the DNA prep was removed, diluted to a concentration of 50µg/ml with 0.5X SSC (final volume 2 or 3 ml) and stored at -20°C in a 15 x 75 mm screw-capped tube for use in mol% G + C determinations. DNA preparations for use in homology experiments were sheared by two passages through a French pressure cell (Aminco, American Instrument Co., Silver Spring, Md.) at 15,000 psi. The preparations were then denatured by boiling for 10 min and placed directly in an ice bath. After cooling, they were centrifuged in 25 ml polypropylene centrifuge tubes at 15,000 rpm for 15 min. at 0-4°C. The DNA preparation was carefully drawn off with a Pasteur pipette, being careful not to draw up any of the "crud" that had pelleted in the tube. The concentration of these preps was then determined by measuring the OD at 260 nm of a 1:20 dilution of the prep in 0.1X SSC. The concentration (mg/ml) was then obtained by multiplying this value by 0.87, which takes into account the increased absorbance of single-stranded DNA. All preparations were then adjusted to 0.4 mg/ml with 0.1X SSC and stored at -20°C in 15 x 75 mm screw-capped "competitor" tubes.

The DNA from reference strains that was to be labeled was prepared in the following way. Thirty µl of the DNA preparation (0.4 mg/ml) was placed into a Microfuge tube (Brinkmann 22 36 430-8) along with 10 µl of 3 M Na acetate, pH 6.0, 60 µl sterile distilled water and 200 µl of ethanol. The tube was then placed in a freezer at -20°C for 1 h. The tube was then spun in the Eppendorf Model 5413 Microfuge for 8 to 10 min and the supernatant was drawn off with a Pasteur pipet. Care was taken to follow the meniscus and not dislodge the DNA pellet. One ml of an ice-cold mixture of 80% ethanol,

20% 0.2 M Na acetate, pH 4.6 was added and the tube placed at -20°C for 15 to 30 min. The tube was then spun and the supernatant drawn off as before. Another brief spin (10 to 15 sec) was performed to collect any residual fluid. The DNA pellet was then dissolved in 50 µl 0.05 M Na acetate, pH 4.8.

Determination of mol% G + C of DNA from selected campylobacter strains

Samples for mol% G + C determinations were dialyzed against 0.5X SSC buffer in the same way as described earlier and frozen in 15 x 75 mm screw-capped tubes until the determinations were performed. A sample of reference DNA, from Escherichia coli strain b, adjusted to a concentration of 50 µg/ml was included. Two hundred ml of the final dialysis buffer (before use) was retained in a 250 ml ground-glass stoppered Erlenmeyer flask in the refrigerator. This buffer was used to wash out the cuvettes between determinations as differences in ionic strength can greatly affect the thermal melting temperature of DNA. The mol% G + C was determined using a Gilford 2400 recording spectrophotometer equipped with a Gilford 2527 thermal programmer. The samples were placed in quartz microcuvettes (Gilford 1242x103) and put into a heating cuvette holder (Gilford 2527-1242x82). The initial temperature was 55°C and the temperature was increased at 1°C per min. The increase in OD at 260 nm or the "thermal melting profile" was recorded on chart paper along with a graphical representation of the temperature increase. The thermal melting point (T_m) was obtained by determining the temperature at which one-half of the increase in absorbance at 260 nm had occurred. The thermal cuvette holder held 4 cuvettes at a time and the reference DNA from E. coli b was used along with 3 test strains in

each determination. The reference DNA (E. coli) was moved around randomly in the holder between determinations to avoid any bias. The mol% G + C for a particular DNA preparation was obtained using the following equation (Mandel et al. 1970):

$$\text{mol\% G + C}_x = \text{mol\% G + C}_{ref} + 1.99\ (T_{m(x)} - T_{m\ ref}),$$

the mol % G + C of the reference strain, E. coli b in this case, being 51. Each preparation was tested 3 times and the average of these determinations was the one reported. All values were rounded off to the nearest whole number, as the accuracy of this method is at best ±1%.

Labeling of reference DNA with ^{125}I

The procedure used for labeling DNA from reference strains is a modification of the procedures of Commerford (1971) and Tereba and McCarthy (1973) as described by Selin et al. (1983). Thirty-five µl of DNA in 0.05 M Na acetate buffer, pH 4.8 was added to 1 ml serum vial (Wheaton 400) along with 2 µl of 7 x 10^{-4}M KI and 3 µL ^{125}I (40 µCI/µL). The vial was then capped and sealed with an Teflon-lined aluminum seal (Wheaton) which was crimped on. One ml of air was removed with a 1 ml syringe and 12.5 µl of $TlCl_3$ (1 mg/ml) was injected into the reaction vial. The $TlCl_3$ is the catalyst and initiates the reaction when added. The vial was mixed and incubated in a water bath at 70°C for 20 min. During this time the ^{125}I forms covalent bonds with the cytosine moieties of the DNA. The reaction mixture was then cooled on ice and 150 µl of 0.5 M phosphate buffer (pH 6.8) was

added to stop the reaction. A second incubation at 70°C was performed to disrupt any weak bonds between the iodine and the DNA. The contents of the vial were then removed with a 1 ml disposable syringe and placed on a Pharmacia PD-10 column which had been equilibriated with 0.14 M phophate buffer (pH 6.8) containing 0.4% electrophoretic grade SDS. The DNA was eluted from the column in a 2 ml volume into a collection tube which also contained 40 µg of sheared, denatured salmon sperm DNA. The column was discarded as radioactive waste after use. The DNA preparation was then heated in a boiling water bath for 5 min and then passed through a Pastuer pipet packed with glass wool and a column of activated hydroxyapatite (DNA grade, Biogel, Bio-Rad) 2.5 cm in length which was equilibriated with 0.14 M phosphate buffer (pH 6.8) containing 0.4% SDS. The Pasteur pipet columns were placed inside of 16 x 125 mm screw-capped test tubes in a 70°C water bath and the elutes collected in the test tubes. After the first gravity elution, the columns were transfered to clean, preheated test tubes. The purpose of the HA column was to remove any double stranded DNA along with any weakly bound ^{125}I. The labeled DNA preparations were then passed through another Pharmacia PD-10 column equilibriated with 0.1X SSC and eluted with 2 ml of 0.1X SSC into a 50 ml centrifuge tube. This step removed the phoshate. The DNA samples were then precipitated by adding 0.2 ml of 3 M Na acetate, pH 6 and 5 ml of ice-cold ethanol. After at least 2 h in a freezer at -20°C, the sample was centrifuged at 12,000 x g for 10 min and the DNA pellet redissolved in 3.3 ml of 0.1X SSC and stored at -20°C until needed.

Buffers and reagents used for DNA homology experiments

The following buffers and reagents were used in the DNA homology experiments.

Buffer A: 0.05 M sodium acetate-0.3M NaCl-0.5 mM $ZnCl_2$, pH 4.6. Stored in a screw-capped 1 liter bottle in the refrigerator.

1.76 M NaCl-10^{-3} M HEPES buffer, pH 7.0: Stored in the refrigerator in 50 ml screw-capped tubes.

1 N HCl mixture for DNA precipitation: 1N HCl, 1% NaH_2PO_4, 1% $Na_4P_2O_7$ (10 H_2). Stored at room temperature in a ground glass stoppered Erlenmeyer flask or in a 1 liter bottle equipped with a Brinkmann Dispensette. A 1:4 dilution of this mixture was used as the HCl wash. This wash mixture was stored in the refrigerator in ground-glass stoppered Erlenmeyer flasks.

S-1 nuclease: An enzyme from Aspergillus oryzae that, under proper conditions, is specific for single-stranded DNA. The enzyme is supplied in glycerol and must be diluted before use. The proper dilution for use is determined by "titrating" the S-1 nuclease (Johnson 1981). In this procedure, two-fold dilutions of the nuclease are prepared and each of these dilutions is incubated with radioactively-labeled, single-stranded bacterial DNA at 50°C for 1 h. The contents of each tube is then acid precipitated, the DNA collected on nitrocellulose membranes and the radioactivity counted in a scintillation or gamma counter. The highest dilution that "chews up" all of the single-stranded DNA, so that no radioactivity can be precipitated is determined. The S-1 nuclease is then used at one-half of this dilution. For example, if a 1:50 dilution will get rid of all of the single-stranded DNA during the incubation period, the S-1 nuclease is used at a dilution of 1:25 in

the homology experiments. The activity of the nuclease should be checked from time to time, to ensure that activity is not lost on storage. A 1:25 dilution of the S-1 nuclease was used for most of the experiments performed during the course of this project. Buffer A (described above) is used to dilute the S-1 nuclease.

Sheared, denaturated salmon sperm DNA : high molecular weight DNA, 0.5 mg/ml, denaturated by boiling for 10 min and sheared by 2 passages through a French pressure cell at 10,000 psi. Stored in 15 x 75 screw-capped tubes at -20°C. Added to the tubes prior to S-1 digestion to ensure that the substrate concentration, in this case, single-stranded DNA, is high enough for the enzyme to be operating at maximum efficiency.

Sheared, native salmon sperm DNA: high molecular weight DNA, 0.4 mg/ml. Sheared and stored as described above. Used in the vials containing only labelled reference DNA, so that the DNA concentration will be high enough to be effectively precipitated by the HCl mixture after S-1 treatment.

DNA homology experiments

The DNA homology experiments were performed in the following manner. Six x 22 mm vials were used for the DNA hybridization. Two tubes were used for each competitor strain, four tubes for the reference strain and four tubes that contained only labeled reference DNA. Ten µl of labeled reference DNA was added to each of the tubes, 75 µl of competitor DNA (0.4 mg/ml) from the appropriate strain was added to each of two tubes (four tubes for the homologous reference DNA) and 75 µl of 0.1X SSC added to the tubes that were to contain label only. Twenty-five µl of 1.76 M NaCl, 10^{-3} M HEPES

buffer was added to all of the tubes. The tubes were capped with modified serum bottle stoppers, vortexed, and placed in a specially made stainless rack designed to hold 120 reaction vials and immersed in a enclosed circulating water bath at 58°C ("optimal" temperature, Marmur and Doty, 1961) for 24 h. The water level was high enough to cover the tubes so that no evaporation from the tubes would occur. When removed, the rack of vials was placed in a freezer at -20°C if it was not practical to continue the experiment. The contents of each reaction vial was removed with a 100 µl Eppendorf pipettor and placed into a 15 x 100 mm polypropylene tube containing 1 ml of Buffer A. Each reaction vial was rinsed out with an additional 100 µl of Buffer A and this was also placed into the polypropylene tube. The same pipet tip was use throughout the procedure. The empty vials and caps were placed into separate beakers of Isoclean. The caps and vials were rinsed out very shortly after use, especially the caps, because Isoclean will corrode them. Next, 50 µl of denatured, sheared salmon sperm DNA was added to each tube. Finally, 50 µl of diluted S-1 nuclease (Calbiochem-Behring Corp., LaJolla, California) was added to each tube, the tubes vortexed and placed in a covered water bath at 50°C for 1 h. After the 1 h incubation, 1 ml of 1N HCl mixture was added to each tube, the tubes vortexed and placed in a refrigerator for 1 h. The acid-precipitated "duplex" DNA was then collected on glass fiber filters in the following manner. A strip of glass fiber filter paper (Whatman GF/F) was placed in a collection device designed to hold 15 tubes (Model CHAP-100, Adaps., Inc., Dedham, Mass.) and wetted with the HCl wash mixture. This device was connected to an aspirator so that a vacuum would draw the liquid down through the filter paper. The device divided the filter paper strip into

15 individual circles and also had a reservoir to hold the radioactive waste. The contents from each of fifteen tubes was poured into each of the 15 holes in the device, each tube rinsed with the HCl wash and allowed to drain in the holes. The tubes were removed and the holes rinsed with the HCl wash. The filter paper was then removed and placed on a tray for drying. After the contents of all of the tubes had been collected, the filter papers were placed under a heat lamp and dried for 1 h. The individual circles were then removed from the filter paper strips, placed into 15 x 100 mm polypropylene tubes and counted for 5 min each in a Beckman 5500 gamma counter. The level of self-renaturation of the label was subtracted off of all of the values and the amount of DNA homology was determined by dividing the amount of radioactivity in heteroduplex form for the competitor strains by the amount of radioactivity in homoduplex form for the reference strain. The radioactivity of the filters was recorded by a teletype attached to the gamma counter. The tape from the teletype was inserted into a reader and the homology values calculated by a computer program and the values printed out using a Wang computer.

Phenotypic characterization of strains

Hippurate hydrolysis. This procedure tests for the presence of the enzyme hippuricase, which hydrolyzes hippuric acid to benzoic acid and glycine. The procedure used here is that described originally by Hwang and Ederer (1975) and modified by Harvey (1980). The production of glycine is demonstrated by the addition of ninhydrin, which combines with the glycine to form a purple color.

Strains were streaked onto FBP agar and the agar plates incubated under 6% O_2, 5% CO_2 and 89% N_2 for 48 h for the catalase-positive strains. The catalase-negative strains were plated onto BF agar and incubated under an atmosphere of 6% O_2, 5% CO_2, 15% H_2 and 74% N_2 for 48 h. A large loopful of inoculum was removed from the plate and suspended in a 8 x 75 mm serum tube containing 0.4 ml of a 1% solution of sodium hippurate. Three tubes were inoculated for each strain tested. The tubes were stoppered with corks and placed in a 37°C water bath for 2 h. Two-tenths ml of ninhydrin reagent (3.5 mg of ninhydrin in 100 ml of a 1:1 (v/v) mixture of acetone and butanol) was added to each tube and the tubes reincubated for 10 min at 37°C. Care was taken not to mix up the tubes. A deep purple color after 10 min reincubation was considered a positive reaction. A light blue or colorless reaction was negative. C. jejuni strain 13136 was an excellent positive control for this test.

Growth in minimal medium (MM). MM is a modification of a defined medium originally described by Smibert (1963). It is useful in distinguishing between C. coli strains (which will grow in MM) and C. jejuni strains (which will not grow in MM).

Twenty-four to 48-h-old semisolid Brucella medium cultures were used as the inoculum and 2 drops from a Pasteur pipette was used to inoculate 5 ml of MM in a 16 x 125 mm test tube. Three tubes of MM were inoculated for each strain tested and the tubes were incubated at 37°C for 3 days. Five ml of semisolid Brucella medium in a 16 x 125 mm tube was used as a control and 3 control tubes were used for each strain tested. All strains were incubated aerobically except the C. mucosalis and C. concisus strains, which

were incubated under an atmosphere of 6% O_2, 5% CO_2, 15% H_2 and 74% N_2. A pellicle of growth after 3 days similar to that occurring in the Brucella medium was considered a positive reaction, a thin haze or no growth was negative. Strains yielding questionable reactions were transferred to fresh MM and reincubated. All strains showing positive reactions were transferred at least once to prove that they could be subcultured in this medium.

DNase activity. Initially, DNase test agar (Difco) supplemented with 0.005% methyl green (Smith et al. 1969) was used to test for DNase activity. However, some strains of C. fetus would not grow on this medium, in which T-soy agar is the basal medium. For this reason, FBP agar supplemented with 0.2% DNA and 0.005% methyl green was used to test for DNase activity in the catalase-positive campylobacters and BF agar supplemented in the same way was used for the catalase-negative campylobacters. Each plate was divided into six areas by drawing a line down the middle and dividing each half into three areas. Each strain was inoculated onto three of the areas in the following manner. A loopful from a 24-48 h old semisolid Brucella medium culture was spread onto each area in a circular spot about 1 cm in diameter. The plates were incubated under 6% O_2, 5% CO_2 and 89% H_2 for the catalase-positive campylobacters and 6% O_2, 5% CO_2, 15% H_2 and 74% N_2 for the catalase-negative campylobacters. After three days, the plates were examined for zones of clearing around the areas of growth. Some strains produced very hazy, indistinct zones, while others produced very distinct zones of clearing. Only those strains that had an average total zone size greater than 3 mm were considered positive. The zones were best measured if

the plates were placed on a white background, such as a piece of typing paper.

Alkaline phosphatase activity. The method used for determining alkaline phosphatase activity is a modification of the method described by Hébert et al. (1982). Strains to be tested for alkaline phosphatase activity were grown aerobically in Roux bottles until a good turbidity was obtained. FBP was the basal medium for the catalase-positive strains (except C. sputorum biovar fecalis) and the CNW strains. BF was the basal medium for the C. sputorum strains and BFF for the C. mucosalis and C. concisus strains. The growth from a Roux bottle was checked for purity by phase contrast microscopy and harvested by centrifugation in a 50 ml polypropylene centrifuge tube at 10,000 rpm at 0-4°C for 10 min. The supernatant was poured into concentrated Lysol and the cell pellet resuspended and washed once in 20 to 30 ml of sterile 0.85% NaCl. After washing, the pellet was resuspended in 0.85% NaCl to a turbidity equivalent to a No. 3 McFarland standard. One-half ml of this cell suspension was placed into a sterile 16 x 125 mm tube and 0.5 ml of the alkaline phosphatase reagent (100 mg disodium p-nitrophenylphosphate in 50 ml of 0.05 M glycine, 5 x 10^{-4} M $MgCl_2$, pH 10.5) added. Three tubes were inoculated for each strain tested. The tubes were placed in a 37°C incubator for 6 h. At the end of this incubation, any visible yellow color was considered a positive reaction. C. jejuni strain 13136 or CNW strain CG-1 served as a positive control and sterile 0.85% NaCl as the negative control.

Aminopeptidase activities. The same cell suspension in 0.85% saline that was used for the alkaline phosphatase determinations was used for the

determination of aminopeptidase activity. Two or 3 drops from a Pasteur pipette was inoculated into a 0.4 ml of a 0.1% solution of the amino acid - ß-naphtylamide in 0.1 M Tris buffer, pH 8.0 (D'Amato et al. 1978). After 6 h incubation at 37°C, 0.2 ml of cinnamaldehyde reagent (p-dimethylaminocinnamaldehyde, 0.15 g; sodium dodecyl sulfate, 25 g; 2-methoxyethanol, 50 ml; glacial acetic acid, 25 ml and distilled water, 925 ml) (API 20S Streptococus system, Analytab, New York) was added. A deep red color indicated a positive reaction. Sterile 0.85% NaCl served as the negative control.

Growth on agar plates containing 1.5% NaCl. Brucella agar supplemented with 1.5% NaCl was used to test for the ability of catalase-positive strains to grow in the presence of 1.5% NaCl. BF served as the basal medium for the catalase-negative strains. This test is based on one proposed by Benjamin et al. (1983) as an important differential characteristic for C. laridis. The only exception is that Benjamin et al. used yeast extract-nutrient agar (YNA) as a basal medium. Plates were streaked with a loopful of growth from a 24 to 48 h old semisolid culture in a continuous streak. The plates were incubated for 2 days under an atmosphere of 6% O_2, 5% CO_2 and 89% N_2 for the catalase-positive strains and 6% O_2, 5% CO_2, 15% H_2 and 74% N_2 for the catalase-negative strains. Unsupplemented Brucella or BF plates were used as positive controls. Three test and three control plates were used for each strain tested. A positive reaction was indicated by growth similar to that occurring on the control plates.

Anaerobic growth in 0.1% trimethylamine-N-oxide (TMAO). A modification of the method described by Benjamin et al. (1983) was used to examine the

ability of strains to grow anaerobically with trimethylamine-N-oxide (TMAO) as a terminal electron acceptor. Anaerobic growth in TMAO is another important differential characteristic for C. laridis strains. Twenty ml portions of MYNA (10 g/l Bacto peptone, 10 g/l Bacto tryptone, 1 g/l yeast extract, 5 g/l NaCl and 2 g/l agar) supplemented with 0.1% trimethylamine-N-oxide dihydrate (Sigma) were dispensed into 20 x 150 mm screw-capped tubes and freshly autoclaved or steamed and cooled to 45°C before inoculation. A 48 h old semisolid culture was used as the inoculum and 4 to 6 drops from a Pasteur pipette were used to inoculate each tube. After inoculation, the caps were screwed down tightly and the tubes were incubated aerobically at 37°C. Growth throughout the tube within 7 days was considered a positive reaction, growth only on the top of the medium was considered negative. Tubes of MYNA without TMAO were included as negative controls. Three TMAO tubes and three control tubes were used for each strain tested. C. laridis strain C729 served as a positive control.

Amino acid decarboxylase activity. Some strains were tested for alanine, arginine, aspartic acid, glutamic acid, glutamine, glycine, leucine, lysine, ornithine and serine decarboxylase acitivities using the following procedure, which is based on a method described by Smibert and Krieg (1981) for the determination of urease activity. Two g of the amino acid, 0.1 g BES buffer and 1 ml of 0.1% phenol red solution were added to 100 ml of distilled water and the pH adjusted to 7.0 with 2N KOH. Two ml volumes were dispensed into 16 x 125 mm tubes and sterilized by autoclaving. One-half ml of the same cell suspension that was used for the alkaline phosphatase and aminopeptidase tests was used as the inoculum. Tubes without the amino acid served as

negative controls. Three tubes with each amino acid and three tubes without any amino acid were inoculated for each strain tested. The tubes were incubated at 37°C and examined after 24 h. A red color was considered a positive reaction.

Sensitivity to brilliant green. Tolerance of brilliant green was proposed by Véron and Chatelain (1973) as a differential characteristic for C. coli. To test for brilliant green tolerance, Brucella agar was supplemented with brilliant green at concentrations of 1:33,000 (0.03 g/l) and 1:100,000 (0.01 g/l). Three agar plates of each concentration of brilliant green and three unsupplemented Brucella agar plates were used for each strain tested. Each plate was inoculated with a loopful of growth from a 24 to 48 h old semisolid culture and the plates incubated under an atmosphere of 6% O_2, 5% CO_2 and 89% N_2 for 5 days. Growth similar to that occuring on the unsupplemented plates was considered positive.

Sensitivity to triphenyltetrazolium chloride. Tolerance of 2,3,5-triphenyltetrazolium chloride (TTC) has been suggested as an important differential characteristic for the catalase-positive campylobacters. It has been reported that C. jejuni and C. coli strains will grow on blood agar plates supplemented with 0.4 g/l TTC, while other catalase-positive campylobacters will not (Skirrow and Benjamin 1980). Earlier, the ability of strains to grow on blood agar plates containing 1.0 g/l TTC was proposed as a means of distinguishing C. jejuni from C. coli strains (Véron and Chatelain 1973). When growth occurs in the presence of TTC, the compound turns a purple-red color because of its reduction to the formazan compound. Brucella agar was used as the basal medium in this study so that the red color would be readily visible.

Brucella agar (490 ml) was dissolved in a 1 liter flask containing a stirring bar and autoclaved. After cooling the basal medium to 45°C, 10 ml of either a 4% or 10% solution of TTC (filter sterilized) was added aseptically to the basal medium, the medium stirred briefly on a magnetic stir plate and poured into plates. Three plates containing each concentration of TTC plus 3 unsupplemented Brucella agar plates were used for each strain. Plates were inoculated with a loopful of growth from a 24 to 48 h old semisolid culture by continuous streaking. The presence of red colonies over more than a third of the plate after 5 days was considered positive. Strains showing scanty growth were repeated.

Catalase activity. This test has been used for almost 30 years as a quick means of distinguishing the pathogenic campylobacters (C. fetus subsp. fetus, C. fetus subsp. venerealis and C. jejuni) found in the reproductive tracts of cattle and sheep from the commensal campylobacters (C. sputorum subsp. bubulus) also found there (Bryner and Frank 1955). One ml of a 3% solution of H_2O_2 was added to a 24-48 h old semisolid culture. The generation of bubbles (O_2 being evolved) indicated a positive reaction.

Oxidase activity. The description of the genus Campylobacter indicates that all campylobacters are oxidase positive (Smibert 1984). This test is supposed to indicate the ability of an organism to use O_2 as a terminal electron acceptor and is indicative of a respiratory type of metabolism. The campylobacters used in this study were tested in one of the two following ways (Smibert and Krieg 1981).

1. A few drops of a 1:1 (vol/vol) mixture of 1% alpha-napthol in 95% ethanol and freshly prepared 1% aqueous dimethyl-p-phenylenediamine oxalate

(Difco) was added to the colonies formed on a Brucella agar plate after 48 to 72 h. If the colonies turned purplish blue within 30 seconds, the organism was considered to be oxidase positive.

2. A piece of filter paper was moistened with a few drops of a freshly prepared 1% solution of tetramethyl-p-phenylenediamine dihydrochloride (Sigma Chemical Co., St. Louis, Mo.). Growth was removed from a Brucella agar plate that had been incubated for 48 to 72 h with a platinum inoculating loop. The growth was smeared on the moistened paper and the development of a purple color within 10 s was considered positive. It is important not to use a regular nichrome loop in this test, because it will give false positive reactions.

Oxidation or fermentation of carbohydrates. Campylobacter species do not utilize sugars either oxidatively or fermentatively (Smibert 1984). To test for the ability of the organisms used in this study to oxidize or ferment carbohydrates, 3 to 4 drops from a Pasteur pipet taken from a 24 to 48 h old semisolid culture was used to inoculate semisolid Brucella medium supplemented with 1% glucose and 0.002% phenol red. If the lower portion of the tube only turned yellow, this indicated fermentation of the glucose. If the upper portion of the tube turned yellow it was indicative of oxidation of the glucose.

H_2S production and Indole production, SIM medium. H_2S production in Sulfide-Indol-Motility (SIM) medium is another useful test for distinguishing the catalase-positive campylobacters from the catalase-negative campylobacters. The catalase-positive campylobacters (with the exception of catalase-positive C. sputorum biovar fecalis strains) do not produce H_2S in

this medium, while C. sputorum, C. mucosalis and C. concisus strains do. This medium was the least sensitive of the three methods used in this study for detecting H_2S production.

Approximately 7 ml of SIM medium (Difco) in a 16 x 150 mm screw-capped tube was inoculated to the bottom with 3 to 4 drops from a Pasteur pipet taken from a 24 to 48 h old semisolid culture. Blackening of the medium throughout the butt after 7 days aerobic incubation (6% O_2, 5% CO_2, 15% H_2 and 74% N_2 for the C. mucosalis and C. concisus strains) was considered positive for H_2S production.

For detecting indole production, 2 ml of xylene was added to the tube (after determining H_2S production after 7 days) and allowed to stand for 2 min. One half ml of Erlich's reagent (1g of p-dimethylaminobenzaldehyde dissolved in 95 ml of 95% ethanol and 20 ml concentrated HCl) was then added. The formation of a pink or red ring in the xylene layer indicated the production of indole, a breakdown product of tryptophane.

H_2S production on TSI slants. TSI agar slants contain ferrous sulfate, which in the presence of H_2S, reacts to form FeS, which can be detected as a blackening of the medium. This medium contains more Fe than the SIM medium and hence, is more sensitive for the detection of H_2S production. Only freshly prepared slants that had a sufficient level of water of syneresis were used.

A loopful of growth from a 24 to 48 h old semi-solid culture was stabbed into the butt of slant and the loop removed and used to streak the slant, all in one motion. The slants, 3 for each strain tested, were incubated under 6% O_2, 5% CO_2 and 89% N_2 for the catalase-positive strains and 6% O_2, 5%

CO_2, 15% H_2 and 75% N_2 for the catalase-negative strains. Incubations were carried out for 7 days and a blackening of the medium was considered a positive reaction. Several types of positive reactions were observed. The C. sputorum strains blackened the entire butt, the C. mucosalis and C. concisus strains formed a diffuse black precipitate throughout the butt, the C. "hyointestinalis" strains formed a black precipitate along the line of the stab, which was much more reproducible if H_2 was included in the atmosphere, and the C. coli strains produced a blackening at the junction of the butt and the slant, reminiscent of the "mustache" formed by Salmonella typhi on TSI slants. The C. coli reactions were most reproducible if there was sufficient water of syneresis present.

H_2S production, Lead acetate strip method. This technique was the most sensitive of the three used in this study for detecting H_2S production. Cultures were inoculated into semisolid Brucella medium supplemented with 0.02% cysteine-HCl and a paper strip impregnated with lead acetate was suspended over the lip of the screw-capped culture tube so that the bottom of the strip was approximately 1 cm above the level of the medium. The tubes were incubated aerobically (except the C. mucosalis and C. concisus strains , see above) at 37°C for 7 days. H_2S production was indicated by a gray or black discoloration of the paper strip due to the production of lead sulfide.

Growth in the presence of 1% glycine, 1% oxgall and 3.5% NaCl. The ability to grow in the presence of 1% glycine is the only laboratory test presently available for differentiating C. fetus subsp. fetus strains from C. fetus subsp. venerealis strains. Growth in 1% oxgall and 3.5% NaCl are useful for

differentiating C. sputorum biovar sputorum from C. sputorum biovars bubulus and fecalis.

The ability of campylobacters to grow in the presence of these compounds was tested by supplementing semisolid Brucella medium with the appropriate compound. In the case of glycine, the pH was adjusted to 7 with KOH. The media were inoculated with 2 to 3 drops from a Pasteur pipet taken from a 24 to 48 h old semisolid culture and incubated at 37°C for 7 days. Unsupplemented Brucella semisolid medium served as a control. A pellicle of growth similar to that occuring in the unsupplemented Brucella medium was considered a positive reaction.

Growth at 25 and 42°C. The ability to grow at 25 or 42°C is useful for differentiating C. fetus from C. jejuni and C. coli strains. In fact, C. jejuni and C. coli strains grow faster 42°C than they do at 37°C, but they will not grow at 25°C. On the other hand, C. fetus strains will grow at 25°C, but rarely at 42°C.

The ability of strains to grow at 25 or 42°C was tested by inoculating semisolid Brucella medium with 2 to 3 drops (Pasteur pipet) of a 24 to 48 h old semisolid culture and placing the media in incubators set at 25 and 42°C for seven days. A pellicle of growth on the surface of the semisolid medium was considered a positive reaction.

Sensitivity to nalidixic acid and cephalothin. Susceptibility to nalidixic acid or cephalothin are other useful tests for differentiating C. fetus strains from C. jejuni and C. coli strains. Resistance to nalidixic acid is also useful for detecting C. laridis strains (Benjamin et al. 1983; Skirrow and Benjamin 1980).

Susceptibility to these agents was tested on Brucella agar plates. The plates were swabbed with a 24 to 48 h old culture, a 30 µg disk of the appropriate compound placed on the center of the plate and the plates incubated under 6%O_2, 5% CO_2 and 89% N_2 for all of the strains except the C. mucosalis and C. concisus strains, which were incubated under 6% O_2, 5% CO_2, 15% H_2 and 74% N_2. Any size zone of clearing around that disk was considered as a "sensitive" reaction.

Nitrate and nitrate reduction. All Campylobacter species reduce nitrate and some reduce nitrite. In fact, some strains have been shown to be able to grow anaerobically with nitrate as the terminal electron acceptor (Goodman and Hoffman 1983). It appears, however, that the basal medium used plays an important role in the ability of these organisms to reduce nitrate past the nitrite stage. Goodman and Hoffman (1983) found that they could grow C. fetus subsp. fetus strains anaerobically with nitrate in a tryptose-based medium but these same strains would not grow anaerobically in Brucella medium supplemented with nitrate.

To test for nitrate and nitrite reduction, we used Brucella medium as the basal medium, because this is the standard basal medium used at the Anaerobe Laboratory for testing the physiological characters of these organisms (Holdeman et al. 1977). Semisolid Brucella medium supplemented with 1.0% KNO_3 was inoculated with 3 to 4 drops (Pasteur pipet) of 24 to 48 h old semisolid culture and incubated for 7 days at 37°C. After incubation, 1 ml of nitrate solution A (1.6 g sulfanilic acid in 200 ml 5N acetic acid) and 0.5 ml of nitrate solution B (1.2 ml of dimethyl-alpha-napthylamine in 100 ml 5N acetic acid) was added. The appearance of a red color after 2 to 5 min

indicated the presence of nitrite in the medium, which in turn indicated that nitrate had been reduced to nitrite. If no red color appeared, then a pinch of powdered Zn was added. The Zn chemically reduces nitrate to nitrite. If a red color appeared after the Zn addition, then the organism was negative for nitrate reduction (residual nitrate in the medium). If the medium remained colorless after Zn addition, then there was no residual nitrate or nitrite in the medium and the organism was positive for both nitrate and nitrite reduction.

The procedure described above was used for testing the catalase-positive strains. The catalase-negative strains were first tested using this procedure, but later the amount of nitrate added to the medium was dropped to 0.7%. for the following reasons. Historically, the catalase-negative campylobacter strains have been described as being able to reduce nitrite (Lawson et al. 1981; Loesche et al. 1965; Smibert 1984). However, when tested in the 1% KNO_3 supplemented medium many of these strains left residual nitrite. The amount of KNO_3 was subsequently lowered, but some of the catalase-negative strains still left residual nitrite in the medium. It is possible that the use of Brucella medium as the basal medium influenced these results (see Chapter 4 and the note above).

H_2 or formate requirement for growth. C. mucosalis and C. concisus strains differ from other campylobacters because they require either H_2 or formate as an electron donor for growth (Lawson et al. 1981; Tanner et al. 1981).

A requirement for H_2 or formate was tested by the following means:

1. Microaerophilic growth was determined on unsupplemented Brucella agar plates incubated either under an atmosphere of 6% 0_2, 5% CO_2 and 89% N_2 or 6% O_2, 5% $C0_2$, 15% H_2. Growth within 7 days was considered positive.

2. Anaerobic growth was determined in pre-reduced anaerobically-sterilized Peptone Yeast Extract Glucose broth with hemin and vitamin K added (PYG-HK). Unsupplemented PYG-HK, PYG-HK supplemented with 0.2% sodium formate and 0.2% ammonium fumarate and PYG-HK supplemented with 0.2% ammonium fumarate alone were inoculated anaerobically with 2 to 3 drops of culture from a Pasteur pipet taken from a 24 to 48 h old semisolid culture and incubated at 37°C for 7 days. Visible turbidity was considered positive for growth.

3. Anaerobic growth was also determined on Brucella agar plates. Unsupplemented Brucella agar plates and Brucella agar plates supplemented with 0.2% fumarate were streaked and placed under an atmosphere of 10% CO_2 and 90% N_2 in a vented anaerobe jar. The jar was flushed exhaustively to remove any residual oxygen. Unsupplemented Brucella agar plates and Brucella agar plates supplemented with 0.2% fumarate were also streaked and incubated under an atmosphere of 15% H_2, 10% CO_2 and 75% N_2.

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Curriculum Vitae

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